A Cytophylogenetic Study of

Pacific Black Flies (Diptera: Simuliidae)

by

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ABSTRACT

The sequential banding patterns of the larval salivary gland polytene chromosomes of seven species of *Inseliellum* (Diptera: Simuliidae) were mapped. This was completed through the comparison with the standard maps of an eighth species of *Inseliellum*, *Simulium cataractarum*. During chromosomal analysis, both fixed and floating inversions were identified. A floating inversion (IIL-1_{ex},2_{ex}) revealed a cytotype within *Simulium exasperans* that is distributed between two islands, Moorea and Tahiti. Inversion data revealed three shared fixed inversions that could be used as phylogenetic characters. In addition, the placement of a chromosomal landmark (the nucleolar organizer, or NO) was used as a phylogenetic character. The result of a cytophylogenetic (transformational) analysis showed two groups: the NO-IL group, and the NO-IS group. A combined phylogeny was created using the published morphological data and the cytological data of the eight species. The combined tree did not differ from the morphological data only tree. Possible routes of dispersal are hypothesized using geological, chromosomal, and phylogenetic data. These data showed a general pattern of dispersal and colonization from older islands to younger islands, with one possible instance of dispersal from younger to older islands. It is postulated that inter-island speciation has allowed this dispersal and colonization, but intra-island speciation has created the diversity seen in *Inseliellum.*

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INTRODUCTION

Black flies (Diptera: Simuliidae) are present virtually everywhere there is fresh running water. These nematocerous dipterans have an extremely wide geographical distribution, and have managed to colonize even the smallest of oceanic islands. The black fly subgenus *Inseliellum* Rubstov is distributed across the South Pacific, more specifically the archipelago of Micronesia, Caroline Islands, Cook Islands, Austral Islands, Society Islands, and Marquesas Islands. All of these islands are inhabited by at least one species of *Inseliellum*, with the Society Islands (specifically Moorea and Tahiti) supporting approximately 25 of the 48 described species of *Inseliellum* (Craig 1997). Since the first description of *Inseliellum* by Edwards (1927), the subgenus has been studied exclusively by D.A. Craig (University of Alberta) over the past 20 years. Morphological and ecological descriptions have been made for the majority of the species, which lead to the formation of a phylogeny of *Inseliellum* by Craig and Currie (1999). However, to answer questions about the dispersal, colonization, and speciation of *Inseliellum*, a biogeography concerning *Inseliellum* was warranted. This biogeography was presented in Craig *et al.* (2001), which included another phylogenetic analysis of *Inseliellum*. Inferences were made on the dispersal and times of possible colonization for species of *Inseliellum* on their respective islands by tracing the rising and moving of these islands from their volcanic hot spots. In addition, ages were given for the earliest possible time that the island could support a black fly fauna (i.e., when the island had fresh running water). The result of these analyses was that *Inseliellum* was similar to the Hawaiian *Drosophila* system, older species occurred on older islands, and as newer islands formed over time, species would jump from one island to another, resulting in

more derived species occurring on younger islands (Craig *et al.* 2001). These conclusions were made from a combination of geological and phylogenetic data (using morphological characters). The Hawaiian *Drosophila* system, however, is based mainly on chromosomal characters. The cytology of *Inseliellum* is poorly known, with only four mentions in the black fly literature (Rothfels 1979b; Craig 1983, 1975; Rothfels 1989).

The black fly larval salivary gland polytene chromosomes have been studied extensively across all simuliid genera through the isolation, photography, and mapping of the banding sections of these giant chromosomes. Through this mapping and comparison of the banding patterns, both intra- and inter-specific (i.e., within species and between species) relationships have been established. The two types of data usually found through banding pattern comparison are inversions and differences in the sex chromosomes. Inversions can appear floating (as polymorphisms) or fixed (always in the homozygous state). Sex chromosome differentiation is detected by the differences in the banding pattern between the X and Y chromosome. Sibling species usually can be identified by their sex chromosomes; such sibling species are morphologically identical but chromosomally different (Rothfels *et al.* 1978). Regarding fixed inversions, if an identical fixed inversion is found in two different species, this inversion can be used as a phylogenetic character, since the probability of two identical chromosomal breaks occurring independently in two different species is very low (Rothfels *et al.* 1978). From these chromosomal data, cytophylogenies can be formed, showing stepwise relationships between species based on chromosomal differences and similarities. The study of polytene chromosomes is now fundamental to taxonomic and faunal studies of black flies (Rothfels 1979a).

Both morphological and chromosomal data have their strengths and weaknesses. However, by using a total evidence approach, one can attempt to combine these different types of data, in an effort to resolve areas that cannot be determined by one type of data alone. Joy and Conn (2001) combined morphological and molecular data in *Inseliellum*, with some success. Chromosomal data and molecular data have also been combined with much success in *Drosophila* (O'Grady *et al.* 2001).

Considering this information, the objectives of this study are as follows:

1. To map the polytene chromosomes of various species of *Inseliellum* to determine both intra- and inter-specific relationships from the banding patterns.
2. To determine any shared chromosomal inversions (fixed and floating) among the various species of *Inseliellum* to create a chromosomal phylogeny (cytophylogeny).
3. Using a combined evidence approach, compare the chromosomal phylogeny to the morphological phylogeny, and then create a combined phylogeny, using both data sets for the species under study.
4. Make inferences about the dispersal of *Inseliellum* using the current biogeography of *Inseliellum* and comparing this to the biogeography of the Hawaiian *Drosophila*.

LITERATURE REVIEW

Black Flies

Black flies form the family Simuliidae, which belongs to the insect order Diptera, or the true flies. These small flies, also known as buffalo gnats, or turkey gnats, are a worldwide family of nematocerous Diptera that are among the most notorious pests of humans, animals, and birds (Kim and Merritt 1987; Crosskey 1990). Black flies are present almost everywhere where there is running water, with the exception of Antarctica and some desert islands. Approximately 1720 species and 25 genera of black flies are described and named (Crosskey and Howard 1997).

Members of the family Simuliidae are best known for the biting habits of the adult females. Blood is a source of nutrient for egg development. This results in the biting habits of most black fly species. The bite is uncomfortable, and possibly causes an allergic reaction in the host (Kim and Merritt 1987; Adler and McCreadie 1997). Black fly attacks impact on humans, poultry, livestock, and wildlife alike (Adler and McCreadie 1997). Transmission of diseases such as human onchocerciasis (river blindness), mansonellosis, avian leucocytozoonosis, bovine onchocerciasis, and probably several arboviruses by black flies, makes them a notable vector of disease, second only to mosquitoes and ticks (Gubler 1998). More than 17.6 million people in tropical Africa and South America are infected by onchocerciasis alone, caused by a filarial worm transmitted by black flies, and 123 million people worldwide are at risk of infection (WHO 1999).

The abundance of black flies causes a reduction in human activities, and promotes economic loss. Even if 'black fly season' only lasts a couple of weeks, forestry,

agriculture, and tourism are severely affected (Crosskey 1990; Adler and McCreadie 1997).

Life Cycle

Similar to the life cycles of other flies, black flies have a four-stage life cycle: egg, larva, pupa, and adult fly (Cranshaw *et al.* 2000). The egg stage may last from under two days to over a year, depending on geography and climate (Crosskey 1990). Larvae hatch from eggs placed on trailing vegetation or other in-stream substrates by mated females (Adler and McCreadie 1997).

Larval growth is characterized by several moults between successively larger substages (instars). The number of larval instars varies from six to nine, depending on the species (Crosskey 1990). After these larval instars, the last-instar larva (pharate pupa) spins a silken cocoon (Stuart and Hunter 1998) and pupates. Through pupation, the silken cocoon remains fastened to the substrate. The pupal stage is thermally dependant and may last from two days to a few weeks. (Adler and McCreadie 1997). When pupation is complete, the adult fly expels air from its respiratory system, and breaks through its pupal cuticle. It then rises to the surface of the water in a bubble of air, and finds a suitable area along the water to dry its wings and cuticle (Adler and McCreadie 1997). Adult life lasts about two weeks. During this time females of most species must mate, acquire a source of sugar for energy, and acquire a blood meal for egg maturation. After a blood meal, females find a suitable aquatic habitat to deposit their eggs (oviposit) (Adler and McCreadie 1997; Cranshaw *et al.* 2000). From chromosomal studies of *Simulium vittatum* the site of ovipositing appears to be the site from which the female originally emerged (natal site) (Rothfels and Featherston 1981). However, mark-

recapture experiments of female *Simulium venustum/verecundum* found that oviposition did not occur at natal sites, but simply in suitable aquatic habitats (Hunter and Jain 2000).

Habitat

The life cycle of black flies is restricted to flowing water , including rivers, creeks, and trickles (Cranshaw *et al.* 2000). Larvae of certain species also thrive in very thin sheets of flowing water (madicolous flow). For example, *Simulium cataractarum* is found in flowing water less than 2.0 mm deep (Craig 1997). In this aquatic environment, larvae and pupae attach themselves using a silk pad to immersed non-living objects or to vegetation (Crosskey 1990). However, only the larvae are mobile, and when disturbed or when the silken pad begins to deteriorate, a larva can relocate itself by looping over the substrate or by releasing its hooklets from the silken pad and drifting downstream, often on a 'life-line' of silk (Crosskey 1990; Adler and McCreadie 1997).

Feeding

In nearly all species larval feeding involves the use of the cephalic head fans (Currie and Craig 1987). Most larvae feed by attaching themselves to the substrate by means of a posterior circlet of hooks, then twist their body 180°. Material is removed from the water by holding the head fans up to the current, thus filter-feeding (Palmer and Craig 2000). An exception *Simulium cataractarum* does not twist its body, and holds its head fans down to the current (Craig 1997). Food consists of five main components: detritus, bacteria, diatoms, filamentous algae, and animal matter.

Certain species of black flies have alternative feeding strategies in which the head fans are reduced or absent. The labral fans of *Crozetia* and some species of *Inseliellum*

are reduced and used for either scraping epilithic algae or browsing rather than filter-feeding (Craig 1977; Currie and Craig 1987). In *Twinnia* and *Gymnopais*, the labral fans are completely absent so that the larvae are obligate browsers (Craig 1974).

Adult black flies feed on both sugar and blood using a highly modified proboscis. For blood, the females proboscis cuts through the skin of a host, and then sucks up the blood (Peterson 1981). Males and females that do not feed on blood have a weak proboscis suitable only for sucking liquids such as water and nectar; the proboscis consists of a labrum, mandibles, laciniae, a hypopharynx, and a labium (Peterson 1981). Biting is exophilic and diurnal (outdoors and within daylight hours), and is usually confined to warm-blooded vertebrates (birds and mammals) (Cranshaw *et al.* 2000). Sugar meals can be acquired from floral nectar, sap, or homopteran honeydew. Sugar meals are taken by males and females of both biting and non-biting species, and have been reported to increase fecundity, and serve as fuel for flight (Adler and McCreadie 1997; Burgin and Hunter 1997a, 1997b).

Flight Range

Flight range of black flies varies greatly with sex and species, since females must usually seek out a blood meal. *Simulium rugglesi* can disperse as little as 3 km, whereas *Simulium damnosum sensu stricto* can disperse as far as 500 km (Crosskey 1990). The degree of dispersal usually depends on whether the species is bloodsucking or non-bloodsucking.

Generation Time

Black fly generation time varies with geography. In habitats with low temperatures and cold winters, there are from two to six generations per season. In lowland tropical rivers that have permanent flow, there may be as many as 16 generations (Craig 1987; Crosskey 1990). In the Marquesas Islands, *Simulium buissoni* has been observed to go through a complete generation in ten days (D. Joy, pers. comm.).

Major Groupings of Black Flies

The family Simuliidae is broken down into two subfamilies, the Parasimuliinae and Simuliinae. Parasimuliinae contains one genus, with two subgenera. Simuliinae contains two tribes, the Prosimuliini, and the Simuliini. Within the Prosimuliini, approximately 21 genera are described, occasionally with subgenera. The tribe Simuliini contains two genera, *Austrosimulium* (with two subgenera) and *Simulium* (with approximately 44 subgenera) (Crosskey 1990).

Geographical Distribution of Black Flies

Black flies occur in the following zoogeographical regions: Afrotropical region (Africa south of mid-Sahara, islands south of southwestern Indian ocean), Australasian region (Australia and Tasmania, New Zealand, Micronesia, Polynesia), Nearctic (North America, Arctic islands, Greenland), Neotropical (South America, Central America), Oriental region (Asia east of Pakistan, central China, Taiwan, east to Sulawesi), Palearctic region (Europe, North Africa, Asia north of Himalayas, British Isles, Japan) (Hubbs 1974). When considering black fly distribution and abundance, these regions are

differentiated by taking into account the numbers of prosimuliines, simuliines, and the number of endemic species.

The Afrotropical fauna contains low numbers of prosimuliines, but holds unique genera in that the larvae and pupae develop on other aquatic arthropods (phoretic black flies). The Australasian fauna consists of only 120 species, due to aridness. This region includes many volcanic islands, with many endemic (Crosskey 1990; Craig *et al.* 1995). The Nearctic fauna contains a high number of prosimuliines in northern areas with numerous species endemic in specific geographic areas. The Neotropical fauna has the most species with several endemic subgenera. The Oriental fauna does not contain any Prosimuliini, and is composed entirely of *Simulium*. The Palearctic fauna is much more speciose in comparison to the Nearctic fauna (Crosskey 1990).

The Australasian region is of interest since it contains many islands both arising from continental land masses and from volcanic activity. This region contains a large number of endemic species of black flies (Takaoka 1996).

Other Dipterans and Black Flies on Islands

Present throughout the Hawaiian archipelago, *Drosophila* (fruit flies) provide excellent examples of dipterans on islands. Volcanic and plate tectonic activity has built up these islands in a process analogous to a moving conveyor belt, with each new island progressively younger than the one immediately preceding it in the chain (Grant 1998). The picture-winged group of *Drosophila* contains 111 species divided into approximately a dozen subgroups based on male genitalia (Grant 1998). Carson (1970, 1974, 1982a; Carson and Yoon 1982; 1983a, 1983b) performed an extensive analysis of inversions in the polytene chromosomes of the 12 subgroups and presented a detailed outline of the

founder species and dispersal of *Drosophila* throughout the Hawaiian island chains. Carson (1982a, 1983a; 1987; Carson and Craddock 1989) then used information about the age of each island to assign a direction to the network of relationships and to produce a biogeographical scheme for the flies. As a result, a pattern of colonization from older to younger island was observed with two exceptions of colonization from a younger to an older island (Grant 1998).

Black flies exist on islands in every ocean, except those that do not have a lotic environment to support a black fly fauna such as Hawaii, Tonga, and Samoa (Crosskey 1990). Islands that have once been part of a continent usually support a black fly fauna that is similar to the nearest continental land mass. However, volcanic islands that are very distant from a continent usually do not have similarities with the black fly fauna found on the nearest continental land mass (Crosskey 1990).

The mechanism of colonization and dispersal to these islands is in question. Introduction by humans is the least probable, since the islands supported a black fly fauna before human colonization. Although adults or immature black flies (larvae or pupae) may have drifted over to the islands on various types of debris, exposure of immature flies to salt water would have been detrimental. Adult flies may have been brought over to the islands by birds (Crosskey 1990). This is the most probable because the majority of species on these islands are ornithophilic (bird biting) (Craig *et al.* 1995). Therefore, mated females had to have been brought over on a bird, because only females blood feed. Finally, winds could have easily carried males and females across great distances over the ocean and drop them onto islands (Craig *et al.* 1995).

Islands of the South Pacific that contain the subgenus *Inseliellum* Rubtsov will be considered further. This subgenus is quite speciose, considering the size of the islands that support its species, having a variety of morphological forms (Craig 1997; Craig and Currie 1999; Joy and Conn 2001).

Pacific Black Flies

Pacific black flies are in the subgenus *Inseliellum*. The distribution range of this subgenus is approximately 9000 km (Craig *et al.* 2001). The geographical distribution of *Inseliellum* is as follows: *Guam*- Micronesia; *Truk, Chuuk*- Caroline Islands; *Rarotonga*- Cook Islands; *Eiao, Nuku Hiva, Ua Huka, Ua Pou, Tahuata, Fatu Hiva, Hiva Oa*- Marquesas Islands; *Bora Bora, Tahaa, Raiatea, Huahine, Moorea, Tahiti*- Society Islands (Craig 1997; Craig *et al.* 2001).

Inseliellum are notorious for their vicious feeding behaviour, both ornithophilic (bird-biting) and anthropophilic (human-biting). This is clearly evident, and is well documented in certain species on the Marquesas Islands, such as *Simulium buissoni*, and *Simulium gallinum* (Craig 1975; Craig *et al.* 1995; Craig 1997; Craig and Currie 1999). In addition, species of *Inseliellum* have an extremely large range of habitat types (Craig 1997).

For example, on Tahiti, larvae (like those of *Simulium cataractarum*) can be found on laminar-flow trickles of water off moss on vertical moss faces. Other habitats include heavily shaded small streams, intensely lit and densely shaded cascades, and thin films of water (madicolous flow) (Craig 1997). Attachment sites for larvae include trailing vegetation, boulders, bedrock, and algae (Craig 1997).

Another unique aspect of *Inseliellum* is that larvae exhibit habitat-specific trophic behaviours. These include filter-feeding with non-twisting of the body, and scraping behaviours (Craig and Currie 1999; Joy and Conn 2001). The labral fans that are used primarily for filter feeding differ immensely among species in both size and ray number, perhaps due to differences in flow and organic matter content in their habitat (Craig and Currie 1999).

Edwards (Edwards 1927) first described three species of Polynesian black flies (*Simulium buissoni, S. cheesmanae,* and *S. tahitiense*), and now there are 48 named species of *Inseliellum*, having been discovered primarily by Craig (1975; Craig and Craig 1986; Craig *et al.* 1995; 1997; Craig and Joy 2000). In addition, Craig (1997), Craig and Currie (1999) and Craig *et al.* (2001) have identified three groups and 1 subgroup based on phylogenetic analyses using morphological characters. As listed in Table 1, the four groups are the tahitiense group, castaneum group, oviceps group, and mesodontium subgroup. In addition, the upper group (all genera above the four groups in the phylogeny) is included in Table 1.

Table 1: Species of *Inseliellum* and which taxonomic group or subgroup they belong to according to Craig (1997; Craig *et al.* 2001). The location of each species is also provided (from Craig *et al.* (2001).

Species	Taxonomic Group	Location
Simulium trukense Stone 1964	Upper	Chuuk
S. guamense Stone 1964	Upper	Guam
S. teruamanga Craig 1983	Upper	Rarotonga
S. rurutuense Craig 1997	Upper	Rurutu
S. gallinum Edwards 1932	Upper	Ua Huka, Ua Poa, Hiva Oa, Tahuata, Fatu Hiva
S. sechani Craig et al. 1995	Upper	Nuku Hiva, Eiau
S. buissoni Edwards 1927	Upper	Ua Huka, Eiau
S. hukaense Sechan 1983	Upper	Hiva Oa, Ua Huka, Nuku Hiva
S. malardei Craig 1983	Upper	All Society Islands
S. lotii Craig 1975	Upper	All Society Islands except Bora Bora
S. arlecchinum Craig 1987	Upper	Tahiti
S. connae Craig 1997	tahitiense	Tahiti
S. dojecorium Craig 1997	tahitiense	Tahiti
S. pseudocorium Craig 1997	tahitiense	Tahiti
S. fossaitae Craig 1997	tahitiense	Tahiti
S. exasperans Craig 1987	tahitiense	Moorea, Tahiti
S. tahitiense Edwards 1927	tahitiense	Tahiti
S. maraaense Craig 1997	castaneum	Tahiti
S. adamsoni Edwards 1932	castaneum	Tahiti

Species	Taxonomic Group	Location
S. pollidicranium Craig and Joy 2000	castaneum	Tahiti
S. hirticranium Craig and Joy 2000	castaneum	Tahiti
S. caesariatum Craig and Joy 2000	castaneum	Tahiti
S. fararae Craig and Joy 2000	castaneum	Tahiti
S. castaneum Craig 1987	castaneum	Tahiti
S. cataractarum Craig 1983	castaneum	Moorea, Tahiti
S. jnabsium Craig 1997	castaneum	Tahiti
S. pufauense Craig 1997	castaneum	Tahiti
S. shannonae Craig 1997	castaneum	Moorea, Tahiti
S. lonckei Craig 1997	castaneum	Tahiti
S. joyae Craig 2000	castaneum	Tahiti
S. bogusium Craig 1997	oviceps	Bora Bora
S. oviceps Edwards 1933	oviceps	Tahiti, Moorea, Raiatea
S. dussertorum Craig 1997	oviceps	Moorea, Tahiti
S. proctorae Craig 1997	mesodontium subgroup	Bora Bora
S. concludium Craig 1997	mesodontium subgroup	Moorea, Tahiti
S. neoviceps Craig 1983	mesodontium subgroup	Moorea, Tahiti
S. clibanarium Craig 1997	mesodontium subgroup	Tahiti

Species	Taxonomic Group	Location
S. mesodontium Craig 1987	mesodontium subgroup	Tahiti
S. middlemissae Craig 1997	mesodontium subgroup	Moorea, Tahiti

Because *Inseliellum* occurs exclusively on archipelagoes formed by hot spots, the mechanism of the dispersal of adult flies by birds or by wind is constantly in question (Craig *et al.* 2001). Regardless, it is important to consider the geographical history of *Inseliellum*, because volcanic islands can be somewhat short-lived, and arising from a hot spot, may move along a fault line over time. Once built up from the sea floor, they emerge above sea level and continue to rise, until the volcanic activity ceases. The actual age of the island is not as important as how long the island has had running water, and thus been black flies (Craig and Joy 2000; Craig *et al.* 2001).

At 20 Ma (20 million years ago), Guam (Figure 1) was available for black fly colonization as were older, now western atolls (eroded volcanic islands) of the Caroline Islands. Molecular evidence indicated that *S. trukense* has been on Chuuk for 14 Ma (Joy and Conn 2001). However, all the other islands now colonized by *Inseliellum* are thousands of kilometers to the south-east. This may indicate that *S. trukense* colonized Chuuk earlier when it was closer south-east, and then drifted westwards (Craig *et al.* 2001).

The formation of the Tonga Islands 20-40 Ma provides an entry for New Caledonia to the east, but it is yet to be determined if black flies are or were present on

the Tonga Islands. In the Cook Islands, only Rarotonga (1.5 Myr.) (million years old) has black flies (Craig and Craig 1986). The islands surrounding it no longer have running water, but they may have been stepping stones for earlier dispersal events (Craig *et al.* 2001). *Simulium teruamanga* and *S. mataverense* occur on Rarotonga, and *S. mataverense* is closely related to the species of the Society Islands. This may indicate that divergence to Polynesia may have taken place 20-10 Ma on the older Cook Islands (Craig *et al.* 2001).

The Marquesas Islands have a limit of 6 Myr. for the time of colonization because older hot spot activity is not indicated. The dispersal of the Marquesasan species is still unclear. In the Society Islands, the first probable habitable islands are 9.4 and 8.1 Myr. old, but are now seamounts (Craig *et al.* 2001). Craig *et al.* (2001) predicted that evolution of species of *Inseliellum* with specialized habitat adaptations took place on Tahiti. Species radiated to large streams (no earlier than 1.5 Ma), and small sunlit rivers (not earlier than 0.87 Ma). Further evidence indicated back-dispersal to Raiatea (not earlier than 1.7 Ma), and to Moorea. Figure 1 represents a summary of the geographical history, focusing on the possible routes of colonization of *Inseliellum* into the Pacific. Much like the Hawaiian *Drosophila* system, older species tend to be found on older islands, and younger species on younger islands. A phylogeny derived from chromosomal data (as in Hawaiian *Drosophila*) may be used to support or refute the proposed dispersal routes.

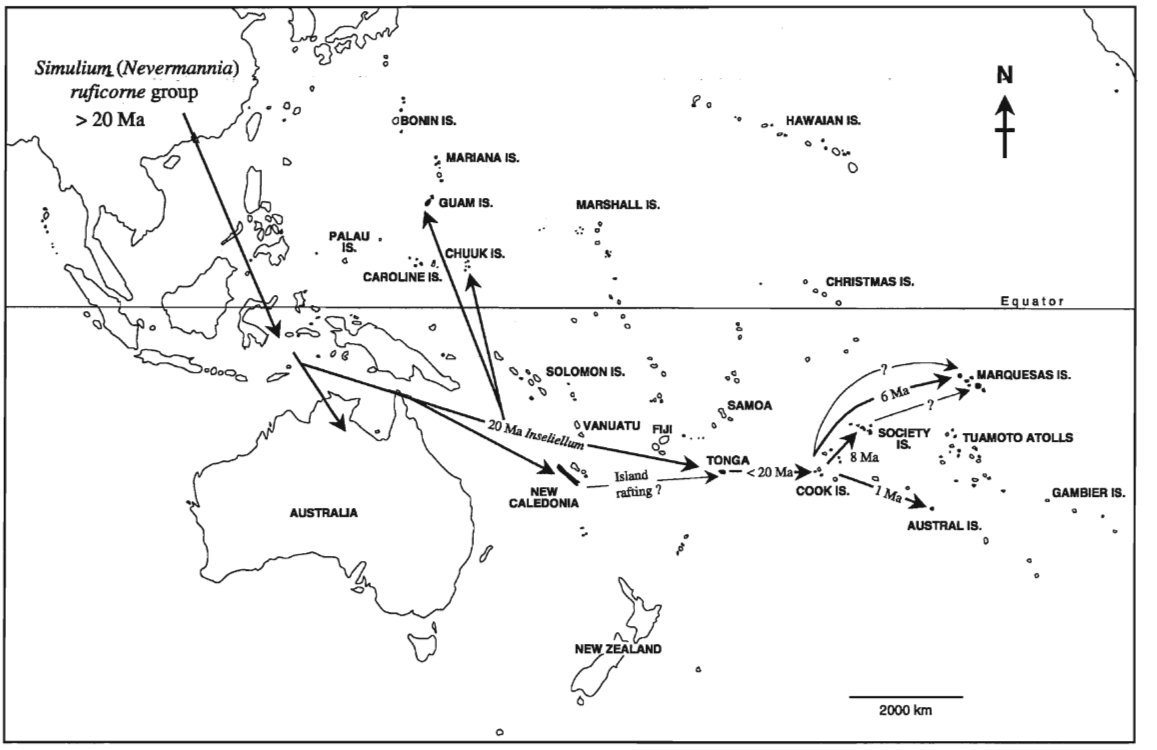

Figure 1: Possible routes of colonization of *Inseliellum* into the Pacific. Question marks indicate alternate routes of colonization. From Craig *et al.* (2001).

Phylogenetic Analyses on *Inseliellum*

The phylogenetic analyses on *Inseliellum* by Craig *et al.* (2001) and Joy and Conn (2001) made inferences on biogeography and dispersal routes. Craig *et al.* (2001) used external morphological data from 48 species of *Inseliellum*. However, their phylogeny also included information on the position of the nucleolar organizer (NO) (a chromosomal character not included in the original data set). The tree was consistent with the hypothesis that the location of nucleolar organizer in a certain chromosome arm was a primative character (NO displaced), and it was assumed that all the species in the upper group had this condition. The rest of the groups in the phylogeny corresponded to their species group designations, had the nucleolar organizer in a different location (NO normal) (Craig and Currie 1999).

Joy and Conn (2001) produced a phylogeny using sequence data from the cytochrome oxidase I (COI) gene, and the small ribosomal subunit (12S) gene for 27 species of *Inseliellum*. The combined data from both genes produced trees with many more steps than the morphological tree, with the 12S gene reducing the overall support of the trees produced (Joy and Conn 2001). Each gene separately resolved certain groups, but incongruence was evident. A total evidence approach (Kluge 1989) using the molecular data and morphological data found only small amounts of congruence between the data sets. The conflict stemmed from the monophyly of taxa having much smaller larval feeding fans (Joy and Conn 2001). Regardless, one major shift in habitat association occurred; cascade and madicolous flow species separated from river and stream species (Joy and Conn 2001).

The total evidence approach used by Joy and Conn (2001) has become commonplace in insect systematics. O'Grady *et al.* (2001) investigated the congruence between chromosome banding patterns of *Drosophila* polytene chromosomes and molecular sequence data. Chromosome inversion phylogenies were less resolved because of fewer characters, but there was a high level of congruence.

Polytene Chromosomes

The giant polytene chromosomes are the only interphase chromosomes that appear as individual structures, a result of multiple divisions of the initial chromatids and their subsequent close pairing (Zhimulev 1996). They are a special case of endopolyploid cells. Endopolyploid cells are cells in tissue that lose the ability to divide by mitosis at a certain time in development. As a result of this loss, the chromosomes

replicate themselves by a process of endomitosis without division of the nucleus. The chromosomes become very thick, and can be observed in interphase.

The first observation of these chromosomes was in 1881, when Balbiani discovered the presence of a faintly stained body appearing as a cylindrical cord that repeatedly unraveled and filled the nucleus in sections of salivary gland cells of larval *Chironomus plumosus* (Chironomidae) larvae (MacGregor 1993; Zhimulev 1996). These bodies were later termed polytene chromosomes in the 1930's.

Polytene chromosomes are 150 - 250 times longer than mitotic chromosomes. They are thick, approximately 2 - 5 μm, and occasionally very thick as 20 μm. DNA content per chromosome within each nucleus correlates with the length of the chromosomes and the number of bands in them (Zhimulev 1999). The nuclei that the chromosomes lie in are in the range of 20 - 100 μm, and a correlation has been established between chromosomes thickness and nuclei diameter (Zhimulev 1996).

The function of polytene chromosomes has been studied extensively, with a certain amount of focus lying on chromosomal 'landmarks' such as the Ring of Balbiani and the nucleolar organizer. Balbiani rings have been shown to be characteristic of simuliid and chironomid polytene chromosomes. These are regions in which genes are in an active state (Zhimulev 1996). The appearance of a specific secretion in an area of the lobe of the salivary gland of *Chironomus pallidivittatus* and the activity of an additional Balbiani ring in the cells of the lobe have been shown to be correlated. The result was that the Balbiani rings of the lobe of the salivary gland code for the major polypeptides of the secretion (Zhimulev 1996).

It has also been demonstrated that the formation of the nucleoli and the functioning of ribosomal RNA takes place in the nucleolar organizer. In addition, both the Balbiani ring and nucleolar organizer puffs have been shown to be actively involved in transcription.

There are different morphological types of polytene chromosomes. The classic polytene chromosome has precise and tight synapsis of the chromatids on homologous regions, and usually occurs in the salivary glands and other organs of dipterans, as well as plants that are grown at low temperatures (that vary in polyteny levels). Another morphological type of polytene chromosome is when the synapsis of the chromatids is weakened, and the chromosomes are loosely pairing. In these types, the banding pattern is retained only in the regions of the largest bands. These types of chromosomes usually occur in some gall midge species, and some plant species such as *Zea mays* (Zhimulev 1996).

Degrees of polyteny may vary from organ to organ. In *Drosophila*, the most distal cells of the salivary gland have the highest degree of polyteny. In addition, temperature has been shown to have an effect on polyteny; lower temperatures stimulate the tighter pairing of the chromatids, whereas higher temperatures weaken the pairing of the chromatids (Zhimulev 1996).

In insects, after completion of embryonic cells, the cells in the majority of organs and tissues do not divide mitotically (except the nervous system and imaginal discs) (Zhimulev 1996). The size of an organ increases as a result of an increase in the size of cells, and consequently their nuclei. This pattern is widespread in different families of Diptera: Sarcophagidae (flesh flies), Chironomidae (midges), Culicidae (mosquitoes),

Muscidae (house fly, stable fly), Calliphoridae (blow flies), Sciaridae (root gnats), and Drosophilidae (fruit flies) (Zhimulev 1996). Endopolyploidy levels reach very high values in insects providing the preconditions for the formation of polytene chromosomes.

Taxonomic interest in polytene chromosomes did not rise until the 1950's, when it was discovered that the differences in the banding patterns could be used to differentiate between species of certain dipterans (MacGregor 1993). The diagnostic feature of polytene chromosomes is their banding patterns. Banding patterns are a result of alternating regions with more compact (chromomeres) and less compact (interchromomeres) regions distributed across each chromatid. When the numerous sister chromatids synapse tightly, homologous chromomeres form a transverse band, giving the banded appearance of polytene chromosomes. The decondensed regions of chromatids between the chromomeres form interbands (Zhimulev 1996).

Black Fly Polytene Chromosomes

All black fly larvae have polytene chromosomes in the nuclei of the cells that compose the walls of the salivary glands. In addition, polytene chromosomes may be found in the gut lining and in the malphigian tubules of adult flies. However, the chromosomes found in the salivary glands have been shown to be the most analyzable (Rothfels and Dunbar 1953; Rothfels *et al.* 1978).

Each polytene nucleus in black fly salivary glands contains three pairs (n=3) of chromosomes (with the exception of some taxa such as *S. aureum,* in which n=2), with the sister chromatids usually being tightly paired with median or submedian centromeres. Centromeres may appear as expanded regions, or in some species, a tight chromocenter is formed (Rothfels 1979b). There is one consistent puff, or landmark within each species

termed the ring of Balbiani, that appears as an expanded region in a section of bands. Each species also has a nucleolar organizer, which forms a large nucleolus (Rothfels 1979b). Other chromosomal landmarks such as the parabalbiani, and the shoe-string can also be used to identify certain chromosome arms. All of these features can be represented diagrammatically on an idiogram, which in turn can be used to diagnose species cytologically (Rothfels 1979b).

Two techniques have been readily used to observe black fly polytene chromosomes. The Feulgen staining method of Rothfels and Dunbar (1953) allows the chromosomes and the reproductive organs of the larvae to be stained in a single step. This method also allows for chromosome preparations to be readily viewed under a light microscope. The lactic/acetic/propanoic acid method potentially gives a higher resolution of bands, but the process is slower, and carcasses have to be re-stained for sex determination (Rothfels and Dunbar 1953).

Black Fly Cytotaxonomy

Cytotaxonomy utilizes chromosomal characteristics in the differentiation of species. The main value or characteristic of black fly polytene chromosomes is in their definitive banding patterns (Rothfels 1979b). Regions of the chromosomes that are not typically banded or densely stained and flaring apart are usually termed heterochromatic. Maps of chromosomes are generated through photography or drawings, and homologous regions between species (interspecific) can be identified. Banding sequences are defined and numbered, and where the sequential homology of a chromosome is broken, as by a heterozygous inversion, reverse pairing in a loop may occur (Rothfels 1979b). Chromosomal inversions or interchanges that occur heterozygously are termed floating

inversions or polymorphisms. The term homosequential refers to identical banding sequences between two species or individuals, but which differ by floating inversions or polymorphisms (Rothfels 1979a). Fixed inversions or interchanges no longer occur heterozygously, i.e., no inversion loops are observed. These inversions may provide taxonomic information. Inversions are the result of two breaks along the chromosome, and subsequent rejoining of the chromosome piece in the opposite direction. Since there is a low probability of two identical breaks occurring independently in two different species, shared fixed inversions may be used as phylogenetic characters (Rothfels 1979b).

Chromosomal rearrangements have led to the determination of the sex-determining locus. In some genera, the sex chromosomes appear undifferentiated (X_0Y_0 system) and the chromosomes appear alike under the microscope (Rothfels 1979b). This condition is considered primitive. However, in many black fly species, the X and Y chromosomes differ in their sequential banding pattern, and quite frequently the initial differences in the sex chromosomes reveal species-specific differences. Differentiation may involve simple or complex inversions, one or more band polymorphisms, or heterochromatinization of larger regions. In nearly all cases, the male is generally the heterogametic sex (Rothfels 1979b).

Previous chromosomal studies have shown many simuliid morphospecies (species that are morphologically distinguishable) comprise of a number of reproductively isolated, biologically distinct sibling species (Rothfels 1981). These sibling species are nearly morphologically identical, but differ chromosomally, usually in the sex chromosomes. Rothfels (1981) and Bedo (1977) explain four ways in which polytene

chromosomes are able to distinguish sibling species. First, banding patterns are unaffected by convergence phenomena, and sibling species differ by fixed inversions or interchanges in one or more of their chromosomes. Second, sibling species may have structurally differentiated sex chromosome sets. Third, inversion polymorphisms may only appear in a homozygous state in one sibling, and heterozygously in another or differ in the frequencies of the standard and inverted sequence. Thus a distinct spectra of intraspecific polymorphisms may be observed. Finally, cytological studies can detect sibling species in sympatry (reproductively isolated from each other) through the absence of hybrids (Rothfels 1981).

The term cytotype is used when existing data (chromosomal, ecological, distributional) are insufficient for assigning species status (McCreadie and Colbo 1993). Cytotypes are identified when inversion polymorphisms are observed more in one form than another intraspecifically. If the inversion polymorphism is not in Hardy-Weinberg equilibrium, cytotype status may be granted on the basis that there is an unequal distribution of homozygotes and heterozygotes for the inversion polymorphism. Cytotypes usually contain a significantly higher number of heterozygotes (thus cytotypes), and inferences can be made on the advantages of being a heterozygote on an intraspecific scale.

Rothfels *et al.* (1978) overviewed the importance of studying polytene chromosomes in black flies. These studies are used foremost to describe populations, siblings, or species in chromosomal terms. On a larger scale, all of the chromosomal information can be used to create cytophylogenies based on the sharing of sequences and subsequent divergence by successive unit rearrangement steps, leading to the

determination of inter-specific relationships. This may be incomplete, since it involves discrimination between similar inversions, stipulations of hypothetical intermediates, and comparisons among sequences that are much scrambled and distantly related (Rothfels *et al.* 1978).

Cytotaxonomic Nomenclature

Rothfels *et al.* (1978) attempted to standardize black fly chromosomal studies by introducing standard chromosomal maps and nomenclature (adapted from Bedo (1977)). In their study, *Simulium venustum* and *S. verecundum* were mapped and these were presented as the standard maps for the subgenus *Simulium.*

The chromosomal complement (n=3) was first numbered in descending order of length as chromosomes I, II, and III. Each arm was labeled a short arm (S) and a long arm (L) in relation to the centromere on the chromosome. The complement was then divided into 100 sections based upon the percentage of the total complement length of each arm. Band sections (not individual bands) were then labeled from the tip of the short arm of chromosome I (IS) (section 1) to the end of the long arm of chromosome III (IIIL) (section 100). Chromosomal landmarks were also identified in an attempt to ease the identification of chromosome arms. These landmarks are in the form of chromosomal puffs or unique band appearances that always occur in a specific chromosome arm. For example the Ring of Balbiani (RB), a puffed region, is always in the IIS arm, and the parabalbiani (PB), and expanded region, is always in the IIL arm (Rothfels *et al.* 1978). Chromosomal inversions were marked on the chromosome maps by brackets, and were labeled in order of their discovery, corresponding to the arm in which the inversion was located (i.e., IIS-1 would be the first fixed inversion discovered in the short arm of

chromosome II) (Rothfels *et al.* 1978). The chromosomal maps provided by this study have been used as a starting point in black fly cytological studies.

Cytophylogenies

Cytophylogenies, chromosomal phylogenies, or cytological transformation series usually accompany black fly cytological studies (Madahar 1969; Bedo 1977; Rothfels *et al.* 1978; Rothfels 1979b, 1981; Rothfels and Featherston 1981; Craig 1983; Bedo 1984; Brockhouse 1985; Hunter and Connolly 1986; Hunter 1987; Conn *et al.* 1989; Rothfels 1989; Ballard and Bedo 1991; Hamada and Adler 1999). These transformation series make inferences about relationships, tracing rearrangements stepwise from a standard sequence (Figure 2) (Rothfels 1979b). From this standard, species are arranged in dichotomies according to shared inversions, interchanges, and nucleolar transpositions. Resolution and the linking of phylogenies are possible, through the detail of linking steps that may still be preserved in other species. The two problems that arise with cytophylogenies are the times of splitting between two taxa (i.e., genetic distances) and the problem with the direction or origin (Rothfels 1981).

The time of splitting would increase as the number of fixed differences between two taxa increases. There is a frequent relation between cytological distance and taxonomic rank. The problem of directionality is analogous to that of the taxonomist who attempts to determine ancestry from compilations of plesiomorphic and apomorphic traits (Rothfels 1981). Despite the fact that standards are rationally chosen, intergeneric inversion differences will defy attempts to define directionality from the cytological data alone (Rothfels 1981).

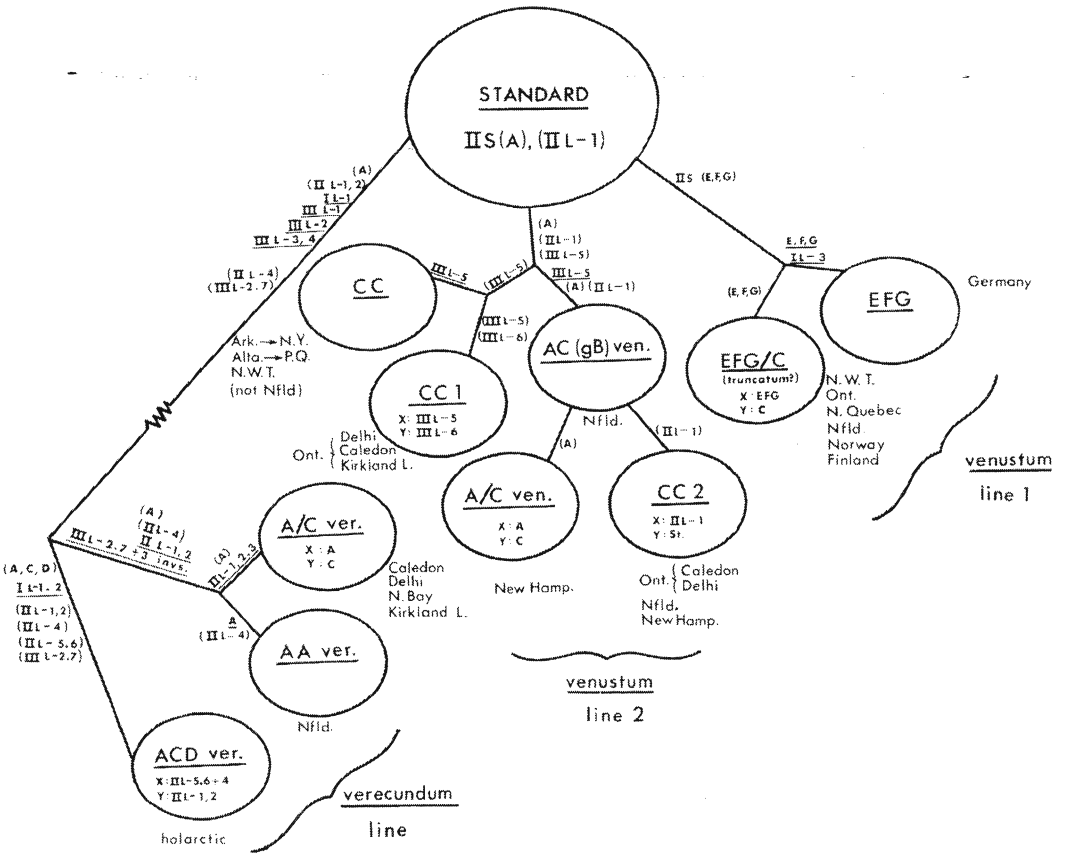

Figure 2: Cytophylogeny (chromosomal transformation series) of some members of the *S. venustum/verecundum* complex. From Rothfels *et al.* (1978).

Studying *Inseliellum* Cytologically

According to Craig (1983), the cytotaxonomy of the Polynesian Simuliidae with emphasis on the Marquesas Islands is of high priority, since any recent attempts have been unsucessful. Only four mentions of cytotaxonomy performed on *Inseliellum* exist in the literature.

In Craig (1975), Rothfels is quoted on two species of *Inseliellum*, *Simulium tahitiense*, and *S. oviceps* as stating, "The two species chromosomally are amazingly close - closer to each other than either two any other Simuliids we have looked at, in fact, there may be basically not more than four inversion differences". This is close to truth, since Rothfels (1979b) commented on *S. tahitiense* and *S. oviceps* as being extremely

close chromosomally - four of the six chromosome arms are identical; no more than four fixed inversions exist, and at least two inversion polymorphisms are still shared. The two species are very similar morphologically, and unique in comparison to other black flies since the females are heterogametic. Female *S. tahitiense* are heterozygous for any of three inversions in IIL, and females of *S. oviceps* are heterozygous for a series of three independent inversions in IIIL. In addition, Rothfels (1979b) reported that *S. oviceps* is male achiasmate, while *S. tahitiense* is male chiasmate.

In Craig (1983), Rothfels distinguished nine cytotypes from Tahiti. These cytotypes were: *Simulium oviceps*, *S. tahitiense*, "IS", "IS complex", "new", "neoviceps", "IIS", "forbidden", and "light". In this study, he denoted "IS" as the standard. Table 2 shows each cytotype and its locality described by Rothfels.

Table 2: The nine species/cytotypes and localities of the Tahitian Simuliids studied cytologically by Rothfels in Craig (1983).

Species/Cytotype	Locality
S. oviceps	Streams and rivers, small current in a few centimeters of water
S. tahitiense	Streams and rivers, small current in a few centimeters of water
"IIS"	Streams and rivers, small current in a few centimeters of water, but only found on Tahiti-iti
"IS"	Small, spring-fed cascades, no waterfalls
"IS complex"	Lac Vaihiria
"forbidden"	Fautaua Valley
"light"	Shaded streams with vegetation, large streams
"new"	Small heavily shaded streams in Tahiti

Craig (1983) reconstructed a cytophylogeny with both chromosomal and morphological characters (Figures 2 and 3). He included additional species of *Inseliellum*, not from Tahiti, including "damn", "teruamanga", "S. gallinum", and *S. buissoni*, all of which had a nucleolar organizer in the long arm of chromosome II (IIL). These species rooted as the sister group to the remaining Tahitian taxa. Neither phylogeny could place "unique" and "forbidden". Unfortunately, the unnamed species in this study have remained unnamed, or perhaps have been named by Craig in subsequent studies. Regardless, the known Polynesian simuliid cytotypes posses heterogametic females, a trait unique only to *Inseliellum* (Craig 1983).

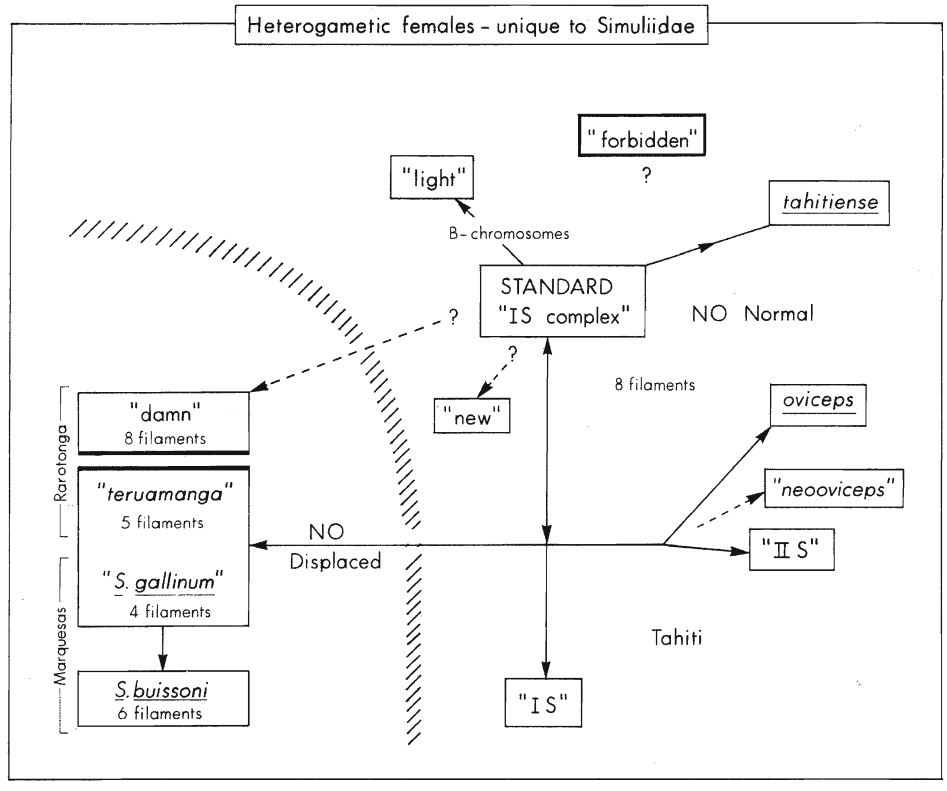

Figure 3: Cytophylogeny of Polynesian Simuliidae by Rothfels in Craig (1983). NO= nucleolar organizer; filaments= pupal gill filaments.

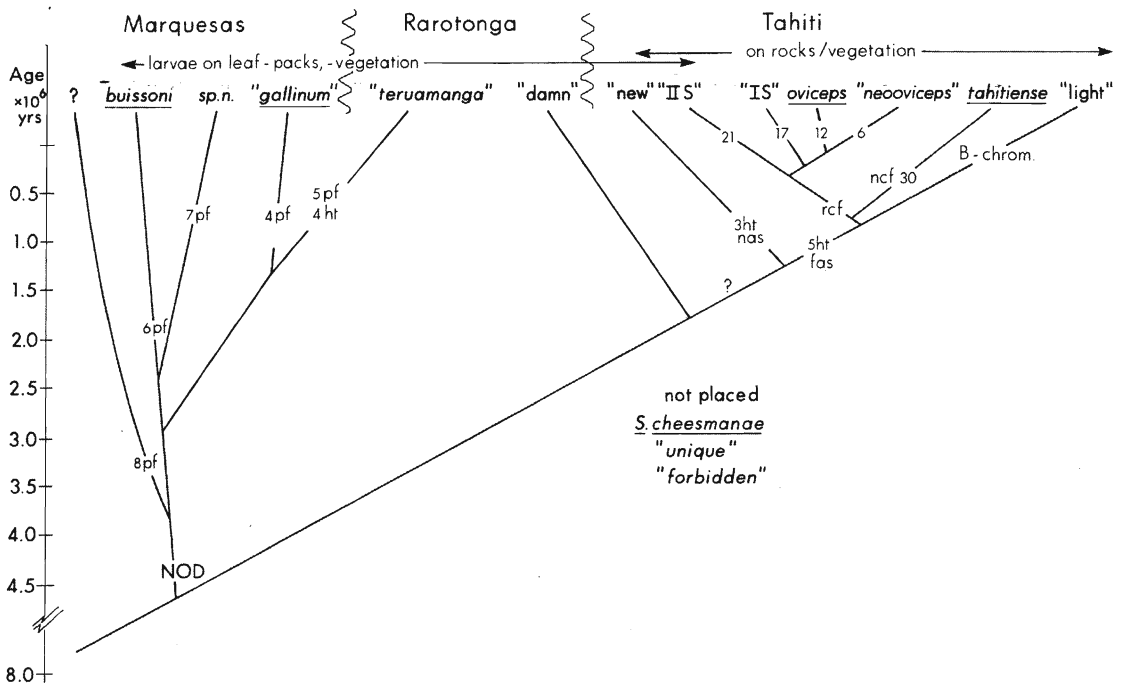

Figure 4: Reconstructed phylogeny for Polynesian Simuliidae. Abbreviations: fas= fused accessory sclerite; ht= number of intermediate hypostomal teeth; nas= normal anal sclerite; ncf= normal cephalic fan; nod= nucleolar organizer displaced; pf= number of pupal gill filaments; rcf= reduced cephalic fan; 12 *ect.*= number of cephalic fan rays. From Craig (1983).

Rothfels (1989) thought the diversity of *Inseliellum* on Tahiti to be the result of one or more founder events on the island. If the population(s) diversified on the island, habitat exploitation could occur through lack of competition. Regarding speciation, Rothfels (1989) stated that the presence of one species on two islands would not exclude sympatric speciation; however, if the species differ as siblings, the argument for sympatric speciation would be strengthened.

This small amount of literature points to the need for a cytotaxanomic study of *Inseliellum*. A complete morphological and molecular phylogeny has been made of *Inseliellum* (Craig *et al.* 2001; Joy and Conn 2001). A total evidence approach could

strengthen or weaken relationships, and give a detailed insight on the biogeography of this subgenus.

MATERIALS AND METHODS

Larval Collections

Larval samples of *Inseliellum* were provided by D.A. Craig (University of Alberta) from four collecting trips to the South Pacific during 1988, 1992, 1998 and 2000. According to Craig (1987, 1997) larvae were brushed off cascades and larger rocks with a shortened shaving brush, stiff enough to dislodge specimens. To collect specimens in rivers or streams, an aquarium net with a mesh size of 0.4 mm was bent to fit the substrate surface downstream to the area that was being brushed in order to capture larvae that were dislodged by brushing. When collecting from vegetation, roots and trailing leaves were placed in a pan full of water until the larvae detached from the vegetation. On average, one hour of collecting time was spent at each locality. Larvae collected for morphological analysis were placed into 95 % ethanol and were studied by D.A. Craig (pers. comm.) Larvae collected for chromosomal analysis were placed into Carnoy's fixative (Appendix A). These samples were stored in plastic-stoppered glass vials, and kept refrigerated at 4°C. All samples that were provided for this study are listed in Table 3.

Table 3: Larval *Inseliellum* samples of provided by D.A. Craig. The collection site and collection date (day-month-year), as well the island in which each site is located on (bold text) is presented. Note: All samples were collected by DAC.

Species	Collection Site (Island)	Collection Date
S. castaneum	Temahani River **(Raiatea)**	2-11-00
S. adamsoni	Hiva Oa **(Marquesas)**	21-10-00
S. dojecoryium	Trois Cascade **(Tahiti)**	11-10-00
S. dussertorum	Belvedre Cascade **(Moorea)**	11-11-00
	1st Afareaitu Cascade **(Moorea)**	11-11-00
S. rurutuense	Above Vaipapa Stream **(Rurutu)**	5-11-00
	Above Hauti Tank Stream **(Rurutu)**	4-11-00
S. lonkei	Vaitamanu Cascade **(Tahiti)**	12-11-00
S. malardei	Belvedre Cascade **(Moorea)**	11-11-00
S. concludium	1st Afareaitu Cascade **(Moorea)**	11-11-00
S. gallinum	Hiva Oa **(Marquesas)**	(19,20,21,24,26)-10-00
S. gallinum	Nuku Hiva **(Marquesas)**	14-10-00
S. buissoni	Nuku Hiva **(Marquesas)**	14-10-00
S. lotii	Trois Cascade **(Tahiti)**	11-10-00
	Fautaua River **(Tahiti)**	10-10-00
	Vaiatarau River **(Tahiti)**	31-10-00
	Haruauta Valley **(Raiatea)**	29-10-00
	Belvedre Cascade **(Moorea)**	11-11-00

Species	Collection Site (Island)	Collection Date
S. exasperans	Jarden Public Vaipahi **(Tahiti)**	9-10-00
	1st Afareaitu Cascade **(Moorea)**	11-11-00
	Belvedre Cascade **(Moorea)**	11-11-00
	Fautaua Cascade **(Tahiti)**	10-10-00
S. tahitiense	Fautaua River **(Tahiti)**	10-10-00
S. cataractarum	Trois Cascade **(Tahiti)**	11-10-00
	1st Afareaitu Cascade **(Moorea)**	11-11-00
	Vaitamanu Cascade **(Tahiti)**	12-11-00
S. arlecchinum	Vaitepiha River **(Tahiti)**	26-03-88
	Tautira Cascade **(Tahiti)**	7-04-88
S. hukaense	Hukamae River **(Marquesas)**	20-07-92
S. guamense	Laelae River **(Guam)**	1-12-98
S. trukense	Winchon Falls **(Chuuk)**	9-12-98

Larvae and Slide Preparation

Depending on the number of larvae available, up to 10 larvae were selected for staining at a time. Mid- to late-instar larvae (except pharate pupae with dark histoblasts that give poor chromosome preparations) were preferred, since larger larvae contain larger salivary glands, and more polytene nuclei.

Using forceps, the 10 chosen larvae were placed in a glass dish containing cold Carnoy's fixative that had been kept refrigerated at 4°C. Each larva was then dissected by splitting it open down the ventral midline, exposing the gut and two salivary glands.

This procedure was done under a dissecting microscope using insect pins attached to wooden applicator sticks handles. After dissection, each larva was placed in a petri dish filled with room temperature distilled water and left for 20 minutes.

While the dissected larvae were in the distilled water, two corked 15 mL glass vials were filled with 5 mL of 1N hydrochloric acid (HCl) and placed in an empty beaker. The beaker was then placed into an incubator preheated to 60°C. When the 20 minute distilled water step was completed, the larvae were placed onto a paper towel to draw off any excess water. Using forceps, they were then quickly placed into the HCl (now at 60°C), and incubated for exactly 9 minutes for mild acid hydrolysis.

Feulgen stain was prepared according to Humason (1967) (Appendix B) prior to larval dissection. After mild acid hydrolysis, the larvae were quickly removed from the acid and placed in another glass vial filled with Feulgen stain at 4°C. This vial was placed into a dark drawer and left for 20 - 45 minutes for staining.

After staining, the larvae were removed from the Feulgen and placed into another vial filled with sulfur dioxide water (SO_2 water) (Appendix C) for 9 minutes in order to remove any precipitated stain. The SO_2 water was then poured off from the vial, and the larvae were rinsed 3 additional times with cold tap water. After rinsing, the glass vial was filled with cold tap water, corked, labeled, and placed in a refrigerator at 4°C overnight.

The next day the larvae were ready for slide preparation. The vial of stained larvae was kept on ice to prevent warming, which tends to affect chromosome morphology. Fisherbrand® Superfrost® Plus 25 X 75 mm slides along with Fisherbrand® 22 X 22 mm cover slips were used to make slide preparations. Each slide

was first labeled in the upper left corner. The slide label included the species, the collection site and date, and the number of the slide. Each slide corresponded to one individual. The sex of the larva was also included, but not recorded until the larva was further dissected.

The slide was placed under a dissecting microscope, and a drop of 50% acetic was placed into the middle of the slide. A stained larva was then placed into the drop of 50% acetic acid. In addition to staining the polytene chromosomes in the salivary glands, Feulgen stain also stains the gonads of the larvae. Testes appeared as two pink dots near the dorsal wall of males, whereas ovaries ran along each side of the gut (Rothfels *et al.* 1978). Thus, males and females were identified and the slide was labeled accordingly. When sex could not be determined, the slide was labeled with a question mark (?).

After sex determination, dissection could proceed. The goal in this dissection was to remove the two salivary glands from the larval body. Stained, the salivary glands appear as tubular structures with pink dots (stained polytene nuclei) (Figure 5) running down the whole length of the body and hooking back up one quarter the length of the body, so that the blind end is near the center of the body, pointing towards the head (Rothfels and Dunbar 1953). The two glands were detected, then plucked or teased out of the larval body using fine forceps. The body was then removed from the drop of acetic acid. Larval bodies were kept in labeled 1.5 mL Eppendorf tubules with 95 % ethanol which were kept in sealed Ziploc® bags. The bodies were then shipped back to D.A. Craig for further morphological study.

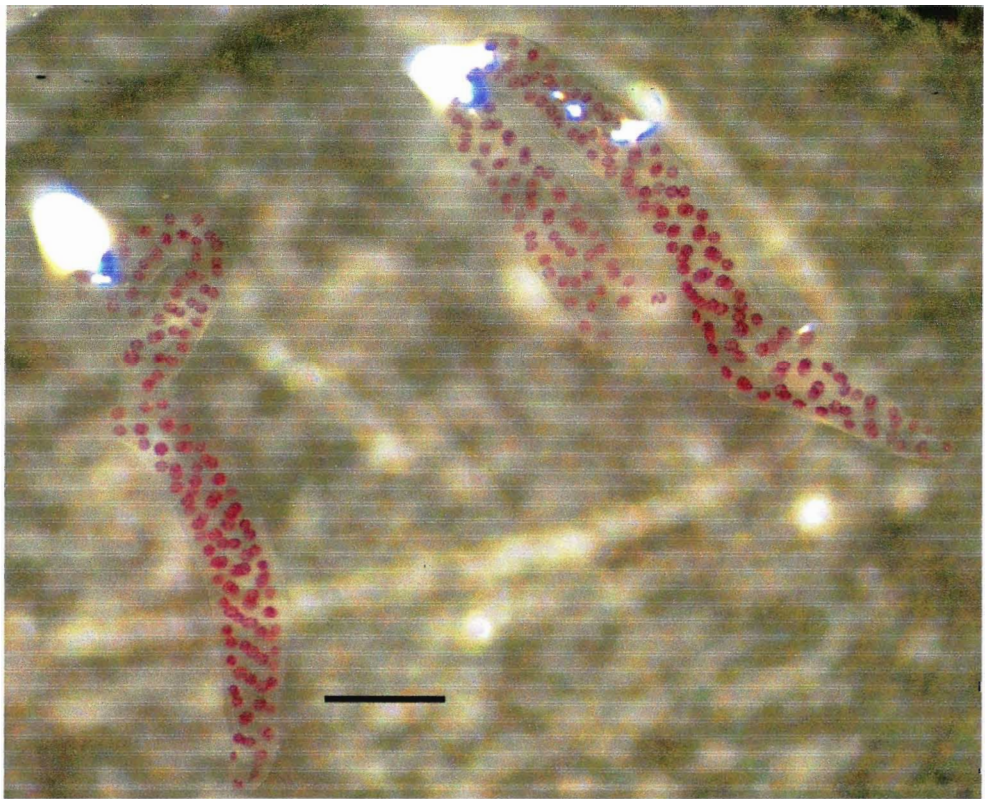

Figure 5: Feulgen-stained salivary glands after dissection showing the polytene nuclei (pink dots) (Magnified 80X, scale bar = 0.13 mm).

With the salivary glands central on the microscope slide, a cover slip was carefully placed on the slide, with care taken not to allow air bubbles to form under the cover slip. Pressing down on the edge of the cover slip with two fingers of one hand, the cover slip was gently tapped from above with the blunt end of an applicator stick with the other hand. This was performed to break open the nuclear membrane of the polytene nuclei to allow the chromosomes to spread out. The slide was placed under a folded piece of paper towel, and using a thumb, the slide was squashed, keeping even and constant pressure on the slide for approximately 3 minutes. After squashing, two drops of 2% aceto-carmine (recipe in Appendix D) were placed at opposite corners of the cover slip to seal around its edges. The slide was then either placed in a slide box and kept in a -80° C freezer, or

observed immediately with a phase-contrast microscope. This procedure was repeated for all larvae in the sample.

Slide Observation and Photography

Slides were observed using a Leitz® DMRE phase-contrast microscope. The slide was observed 10X - 40X power to check for the quality of the preparation. Slide quality (excellent to poor) was noted and recorded.

High-quality slides were re-observed and possibly photographed. Good nuclei were observed under oil immersion (100X objective). Chromosomes were photographed if the bands still appeared clear and the majority of the complement (n=3) was intact. Photographs were taken using a Leica® DC 300 digital camera mounted on the phase-contrast microscope. All images were captured using Leica® TWAIN software, and then imported into Photoshop 6 (Adobe Systems, San Jose, CA) for final editing and subsequent printing.

Pre-Photo Analysis Preparation

Before the banding patterns were mapped, each chromosome arm was identified. Chromosome maps from *Simulium cataractarum* were used as guidelines in identifying the three chromosome arms (Spironello *et al.* in press, maps reproduced in Figures 8-13).

Chromosome Analysis

Chromosomal maps of *Simulium cataractarum* were used as the standard chromosome maps against which all the species in this study were compared to Figures 8-13. The chromosome complement in the standard maps has been divided into 100 sections, with each arm containing the number of sections based upon its percentage total

complement length (% TCL). Chromosome sections were numbered from the short arm of chromosome I (IS) to the long arm of chromosome III (IIIL) as outlined in Rothfels *et al.* (1978) (Figures 8-13).

Band sections in the standard were adhered to when identifying sections in the other species under study. In homosequential regions, the band sections appear in numerical order, in contrast to regions where inversions may not appear in numerical order, but rather in reverse order, or as an inversion loop. Inversions were recorded with forward slashes that denoted where the numerical order was broken. For example, the IIS arm in *Simulium arlecchinum* was written as 43-46/51-47/52-54. Therefore, the band sections 47 to 51 have been flipped at the breakpoints 46/47 and 51/52 to give the banding sequence 43-46/51-47/52-54. It should be noted that when identifying band sections and inversions (especially inversion breakpoints) the chromosome was observed under oil immersion to detect additional detail that photographs may not have had.

Brackets were used on photographs to indicate inversions. Solid brackets were used for labeling fixed inversions, while faded brackets were used to label areas where inversions overlapped. The brackets were then labeled with the name assigned to the inversion (Figures 14 and 19).

Fixed and floating inversions were named according to two different schemes. Fixed inversions were underlined, and showed the arm in which the inversion was located and the name of the inversion number (order of discovery). For example, <u>IIIL-1</u> is the first fixed inversion relative to the standard. Floating inversions were named within each species in the order of their discovery, they were not underlined and they included a species-specific epithet. For example, IIL-1_{ar} was a floating inversion in the IIL arm of

Simulium arlecchinum. Numerical ordering was restricted to the chromosome arm, thus another floating inversion in IIL would be IIL-2_{ar}, but a floating inversion in IL would be IL-1_{ar}. For this study, the epithet used in all cases was the first two letters of the species name in which the floating inversion was found. Complex floating inversions (i.e., having more than one rearrangement) in which the rearrangements occur in tandem were listed in their numerical order separated by a period, for example IL-1_{ex}.2_{ex}. Complex floating inversions in which the rearrangements overlapped each other were listed in their numerical order separated by a comma, for example IIL-1_{ex},2_{ex} (Bedo 1977). Floating inversions were identified as being either homozygous or heterozygous in comparison to the standard (i.e., standard/standard, inverted/inverted, or standard/inverted).

For fixed inversions, the order of the banding sections was compared to determine any shared inversions. For example, if Species A had a band sequence in IIS written as 43-45/48-46/49-54, and Species B had a band sequence in IIS written as 43-45/51-49/46-48/52-54, an inversion would be noted as shared between the two (sections 46-48). The sequence would then be written in numerical order, but including the breakpoints from both species: 43-45/46-48/49-51/52-54. Then the sequences were reversed at the breakpoints to fit the sequence in each species. Every time a sequence was reversed, this was noted as an inversion step. Thus, if the previous sequence was reversed at 46-48, the resulting sequence would be 43-45/<u>48-46</u>/49-51/52-54. This sequence is Species A, and only took one inversion step. Species A would be designated as having the inversion <u>IIS-1</u>. If this process was continued, and the sequence 48-46/49-51 was reversed, the sequence would be 43-45/<u>51-49/46-48</u>/52-54. This sequence is Species B, and took an additional or second inversion step. Species B would be recorded as having a complex

overlapping inversion, named IIS-1,2. If a fixed inversion was unique and not shared between any species, it was named as it was discovered. For this example, if there was another species that had an inversion in IIS, but different than either of the inversions found in Species A and B, it would be named IIS-3. This method was used to determine and name all fixed inversions for this study.

Idiograms (Figure 7) were created to show the entire complement of each species with landmark and inversion locations diagrammatically.

Intra-Specific Analysis

Chromosomes of males and females from each site were scored for each inversion and the state that each inversion was in (i.e., homozygous or heterozygous). These data were used to determined sex-linkage, and thus possible male or female heterogamety. In addition, the frequencies of the standard and inverted sequences of each inversion were calculated and used in a Hardy-Weinberg calculation. These expected values were compared to the observed numbers in the population, and a G-statistic (Zar 1984) was used to determine any significant differences between the values (i.e., if the population was in Hardy-Weinberg equilibrium for the particular inversion).

Inter-Specific Analysis

Any shared fixed inversions were considered as taxonomic characters. The position of the nucleolar organizer was also used as a character, and a cytophylogeny (cytological transformation series) was formed.

Informative morphological characters from the Craig *et al.* (2001) data matrix were determined for the species used in the study and for the outgroup. These characters

were placed into a data matrix using MacClade (ver. 3.05). The data matrix was analyzed using PĀUP (Swofford) (ver. 3.1.1). Trees were created using a heuristic search with the default settings of the program. Character changes on branches, consistency index (CI) and retention index (RI) were noted for each tree. A consensus tree using the 50% majority rule was used for decay index determination. Decay index (Bremer 1988) for clades was calculated by performing an exhaustive search using PAUP, but restricting the output tree length to one number higher than the tree length of the 50% majority rule tree. This tree was observed for any clades from the original 50% majority tree that have collapsed into polytomy. This method was then repeated, noting how many additional steps it took for each clade to collapse.

Chromosomal characters were coded (0 to 1) and added to the morphological data matrix. Character states for the outgroup were coded as question marks (?). The combined data matrix was evaluated the same as the morphological data matrix. Molecular characters from Joy and Conn (2001) were not used in the combined analysis because the molecular data matrix was not usable, and certain species in this study were not used by Joy and Conn (2001).

The nucleolar organizer character was reweighted in the combined phylogeny because Craig and Currie (1999) and Craig *et al.* (2001) used this character to define the two major clades in *Inseliellum*. The nucleolar organizer was thus weighted from 2 to 10.

The groupings of species were compared between the morphological and combined phylogenies, with the cytophylogeny taken into consideration.

RESULTS

Collection

The collections provided by D.A. Craig resulted in 18 species of *Inseliellum* from 17 different sites (Table 3). Slides were prepared for 17 species. When possible, ten slides were made each time the sample was used. In total, 314 slides were made (Table 4). Seven species yielded chromosome data at a quality that allowed the banding patterns to be mapped, including *Simulium arlecchinum, S. exasperans, S. dussertorum, S. lotii, S. hukaense, S. buissoni, S. rurutuense.* Table 5 lists the seven species used in the study, the sites from which they were collected, and a breakdown of the number of sexed individuals. Larval heads (except *S. hukaense*) are shown in Figure 6.

Table 4: Slides of *Inseliellum* prepared for this study (minus *S. cataractarum*- refer to footnote).

Species	# of Sites Sampled	# of Slides Prepared	# of Scorable Slides
Simulium arlecchinum	2	7	7
S. exasperans	4	43	31
S. dussertorum	1	5	5
S. lotii	2	14	9
S. hukaense	1	10	10
S. buissoni	1	4	4
S. rurutuense	2	41	24
*S. cataractarum**	5	72	63
S. dojecorium	1	34	5
S. adamsoni	1	5	2
S. trukense	1	19	2
S. guamense	1	5	0
S. tahitiense	1	28	1
S. lonkei	1	3	2
S. malardei	1	1	1
S. concludium	1	1	1
S. castaneum	1	22	0
Total	27	314	167

*these slides were prepared for Spironello *et al.* (unpublished) during the initial course of this study

Table 5: The seven species of *Inseliellum* used for chromosomal analysis in this study arranged by site and collection date (day-month-year). Numbers of males, females, unknowns, and totals are given.

Species	Site	Collection Date	Males	Females	Unknown	Total
S. arlecchinum	Vaitepiha River	26-03-88	0	1	3	**4**
	Tautira Cascade	7-04-88	0	3	0	**3**
S. exasperans	1st Afareaitu Cascade	11-11-00	5	5	3	**13**
	Jarden Public Vaipahi	9-10-00	1	5	4	**10**
	Belvedre Cascade	11-11-00	2	4	2	**8**
S. dussertorum	Belvedre Cascade	11-11-00	1	1	3	**5**
S. lotii	Raiatea, Haruauta Valley	29-10-00	2	6	1	**9**
S. hukaense	Hukamae River	20-07-92	5	3	2	**10**
S. buissoni	Nuku Hiva	14-10-00	3	1	0	**4**
S. rurutuense	Above Hauti Tank Stream	4-11-00	2	6	7	**15**
	Above Vaipapa Stream	5-11-00	1	7	1	**9**

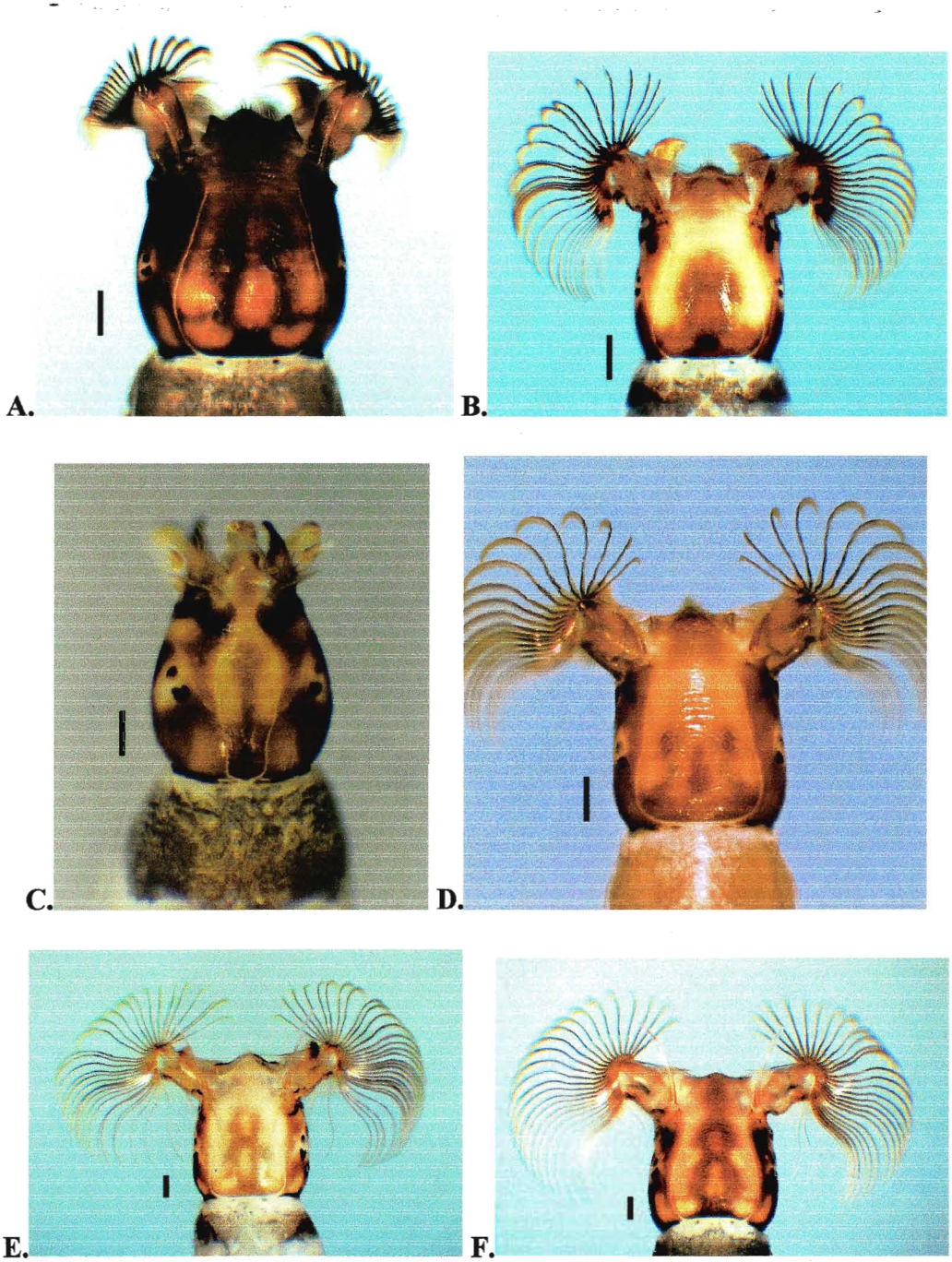

Figure 6: Larval head photographs (dorsal side) of six of the seven species of *Inseliellum* used for chromosomal analysis in this study. A- *S. arlecchinum*, B- *S. exasperans*, C- *S. dussertorum*, D- *S. lotii*, E- *S. buissoni*, F- *S. rurutuense*. Absent is *S. hukaense*. Photographs courtesy of D.A. Craig from Craig (1997; Craig and Joy 2000; Craig *et al.* 2001). Scale bar = 0.1 mm.

Intra-Specifc Analysis

General Observations

All species had 3 chromosomes (2n=6) with the homologues tightly paired. No species contained chromocenters. All chromosomal landmarks were visible and the location of each could readily be detected. Idiograms for each species were made and are shown in Figure 7. The only chromosomal landmark that varied in its position was the nucleolar organizer (NO), which was in the base of IS in the *S. cataractarum* standard and in *S. arlecchinum*, *S. exasperans*, *S. dussertorum*, and *S. lotii*, and in the base of IIL in *S. hukaense*, *S. buissoni*, and *S. rurutuense*. Relative positions of the Ring of Balbiani landmark (section 46 of IIS), parabalbiani landmark (section 64 of IIL), the bulge landmark (section 49 of IIS), and the blister landmark (section 77 of IIIS) are shown in Figure 7 for each species. Centromeres were not always well defined, showing inconsistency in their definition. The most defined centromere was usually seen in chromosome I in species that had the NO in IS. Chromosomes I and II were found to approximately metacentric (centromere located centrally), while chromosome III was consistently sub-metacentric (centromere not located centrally). Chromosome arm lengths were relatively similar, except for a highly condensed region in IIL in *S. dussertorum* (Figure 7C). Inversions did not affect chromosome arm lengths. The most complex floating inversion was found in the IL arm of *S. dussertorum*, in which the inversion took up the majority of the arm through a series of inversion loops. The numerical banding sequence of each chromosome arm in comparison to the *S. cataractarum* standard is given in Tables 6a and 6b. The standard maps of *S. cataractarum* are given in Figures 8-13.

Figure 7: Idiograms for the seven species under study and the standard S. cataractarum maps. A- *Simulium cataractarum*; B- *Simulium exasperans*; C- *S. dussertorum*; D- *S. lotii*; E- *S. hukaense*; F- *S. rurutuense*; G- *S. buissoni*; H- *S. arlecchinum*. Chromosomes are numbered I to III (left to right), with S denoting the short arm and L denoting the long arm. Centromeres are indicated by horizontal line on the chromosome arms. Fixed inversions are labeled and indicated by brackets on the left of the chromosome arms, while floating inversions are labeled and indicated by brackets on the right of the chromosomes. NO- Nucleolar Organizer; RB- Ring of Balbiani; B- Bulge; PB- Parabalbiani; BL- Blister. The polarity of the RB is given by a horizontal line above or below the landmark, and the polarity of the Blister is given by the direction of the "U" (pointing up or down).

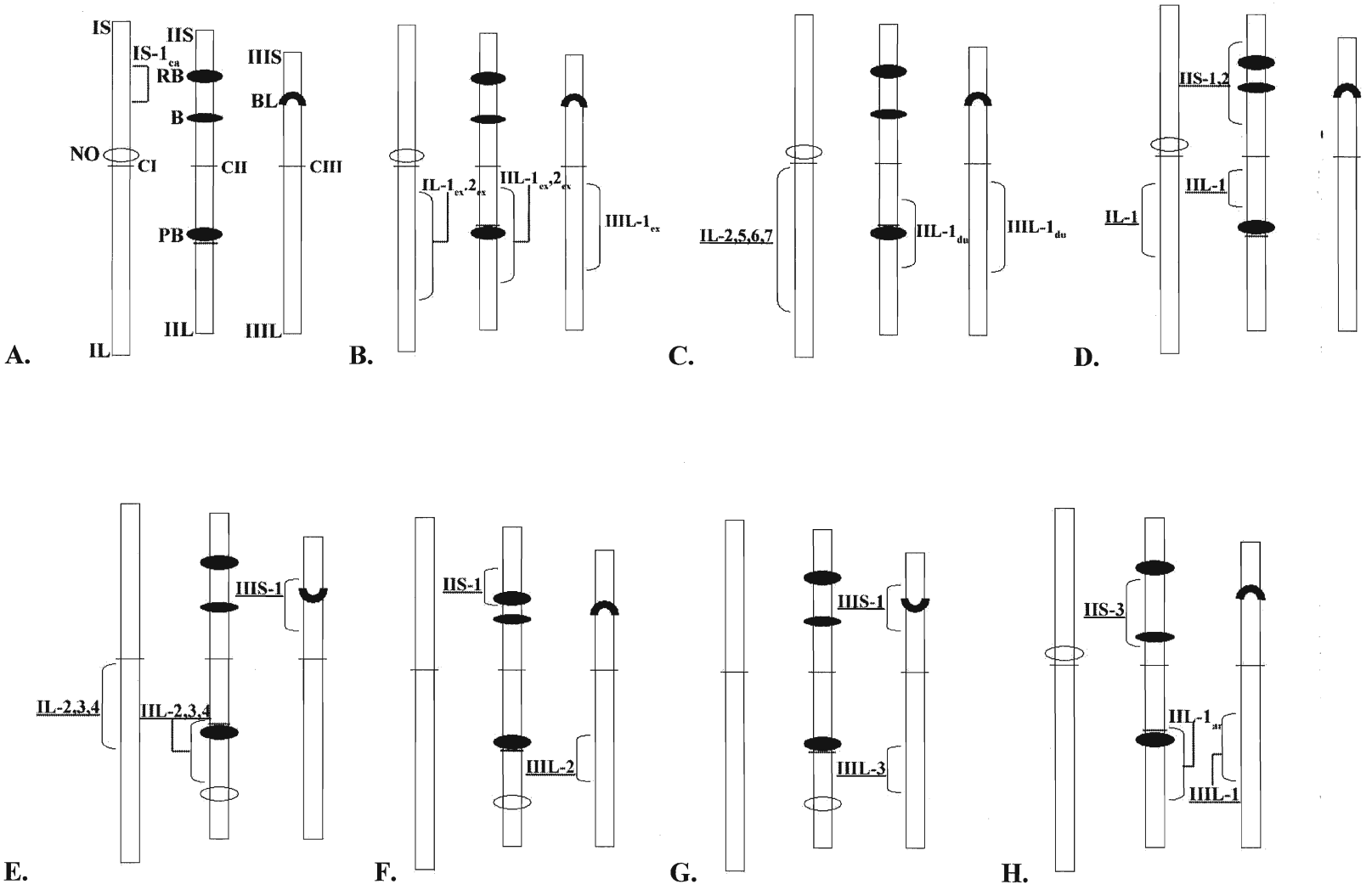
IS
IIS
IS-1ca
RB
B
NO
CI
CII
PB
IIL
IL
A.
IIIS
BL
CIII
IIIL
IL-1ex,2ex
IIL-1ex,2ex
IIIL-1ex
B.
IL-2,5,6,7
IIL-1du
IIIL-1du
C.
IIS-1,2
IIL-1
IL-1
D.
IIIS-1
IL-2,3,4
IIL-2,3,4
E.
IIS-1
IIIL-2
F.
IIIS-1
IIIL-3
G.
IIS-3
IIL-1ar
IIIL-1
H.

Table 6a: Numerical banding sequences of each chromosome arm of the seven species under study in comparison to the standard, *Simulium cataractarum*. Breakpoints of inversions are denoted by forward slashes.

Arm	*S. arlecchinum*	*S. exasperans*	*S. dussertorum*
IS	1-20a	1-20a	1-20a
IL	20b-42	20b-24/31-25/39-32/40-42	20b/26-25/21-24/27-32/35/33-34/36-42
IIS	43-46/51-47/52-54a	43-54a	43-54a
IIL	54b-59/64-60/65-71	54b-57/68-66/63-65/62-60/59-58/69-71	54b-55/65-56/66-71
IIIS	72-82a	72-82a	72-82a
IIIL	82b-84/95-85/96-100	82b-86/94-87/99-100	82b-87/98-88/99-100

Table 6b: Numerical banding sequences of each chromosome arm of the seven species under study in comparison to the standard, *Simulium cataractarum*. Breakpoints of inversions are denoted by forward slashes.

Arm	*S. lotii*	*S. hukaense*	*S. buissoni*	*S. rurutuense*
IS	1-20a	1-20a	1-20a	1-20a
IL	20b-24/28-25/29-42	20b/25-31/21-24/32-42	20b-42	20b-42
IIS	43-45/51-49/46-48/52-54a	43-54a	43-45/48-46/49-54a	43-54a
IIL	54b/59-55/60-71	54b-59/67-66/63/60-62/65-64/68-71	54b-71	54b-71
IIIS	72-82a	72-75/77-76/78-82a	72-82a	72-75/77-76/78-82a
IIIL	82b-100	82b-100	82b-89/92-90/93-100	82b-88a/94a-88b/94b-100

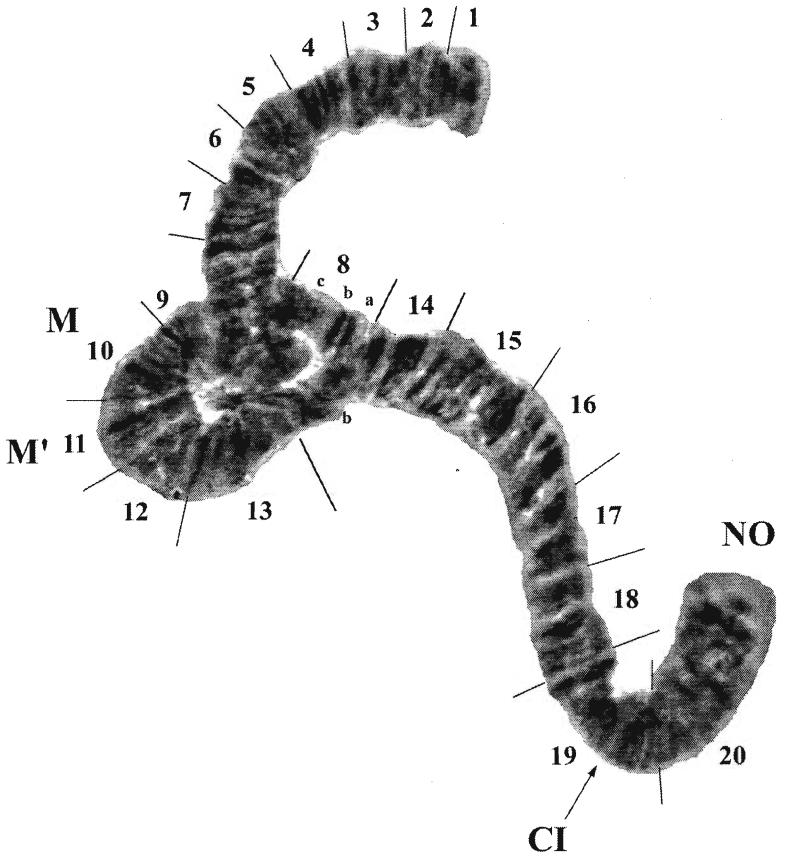

Figure 8: IS arm of *Simulium cataractarum* used as the standard map. M= marker, NO= nucleolar organizer, CI= centromere.

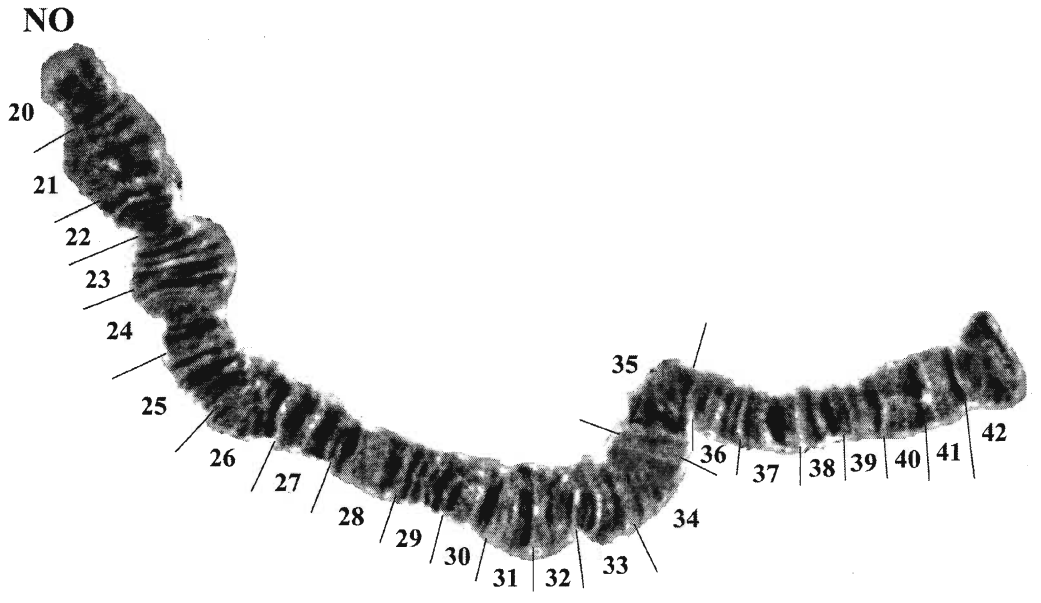

Figure 9: IL arm of *Simulium cataractarum* used as the standard map. NO= nucleolar organizer.

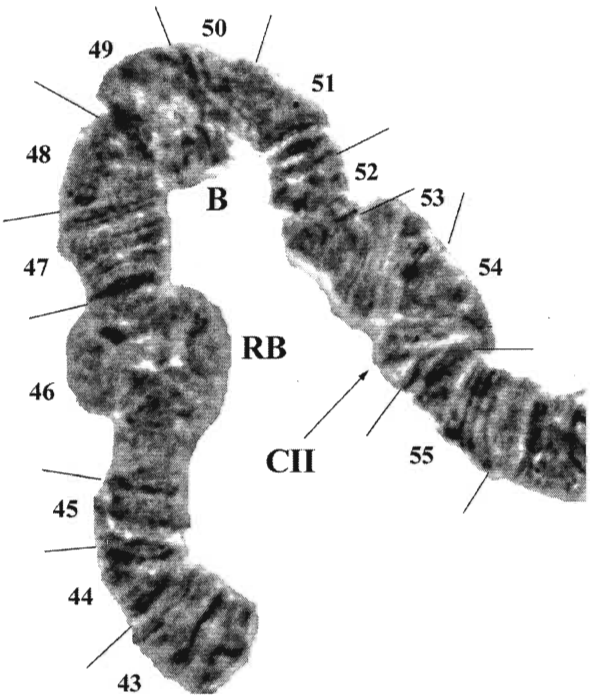

Figure 10: IIS arm of *Simulium cataractarum* used as the standard map. B= blister, RB= Ring of Balbiani, CII= centromere.

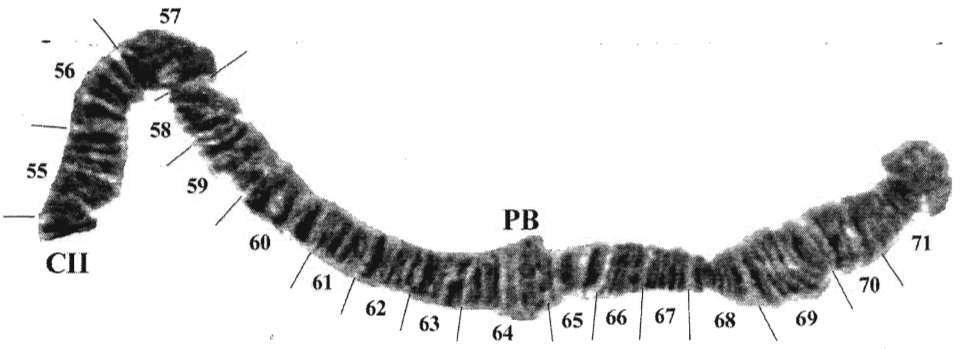

Figure 11: IIL arm of *Simulium cataractarum* used as the standard map. CII= centromere, PB= parabalbiani.

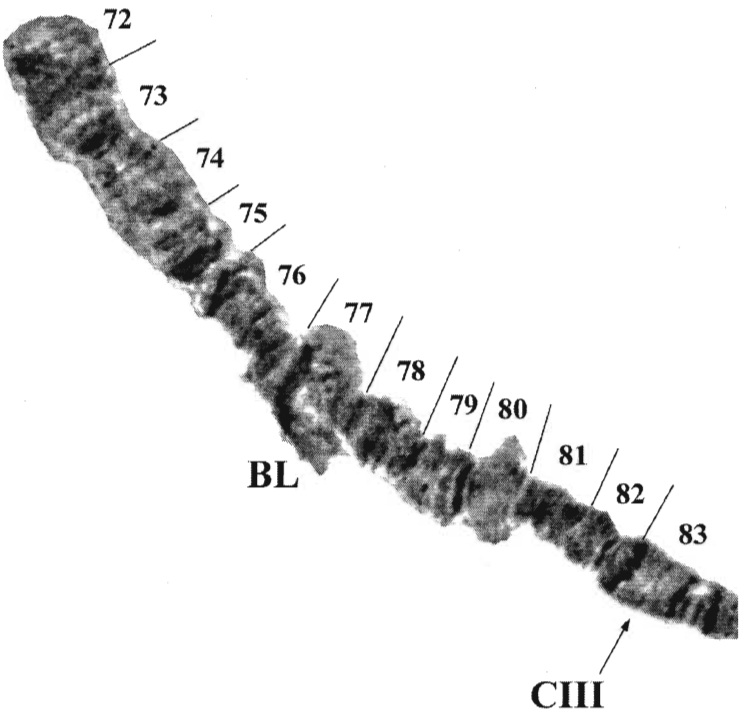

Figure 12: IIIS arm of *Simulium cataractarum* used as the standard map. BL= blister, CIII= centromere.

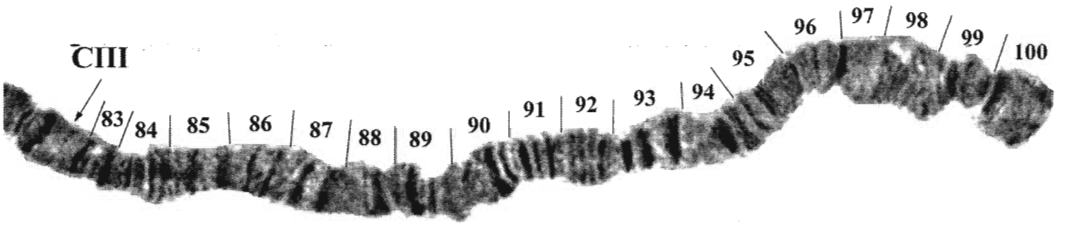

Figure 13: IIIL arm of *Simulium cataractarum* used as the standard map. CIII= centromere.

Simulium arlecchinum

Chromosome I (both long and short arms) and IIIS were homosequential to the *S. cataractarum* standard. Fixed inversions existed in IIS (IIS-3 inversion) (Figure 14), IIIL (IIIL-1 inversion) (Figure 16), and a floating inversion polymorphism existed in IIL (IIL-1_{ar}) (Figure 15).

Larvae of *Simulium arlecchinum* were sampled at two sites- Vaitepiha River and Tautira Cascade (Tahiti) (Table 5). Only 4 and 3 individuals were collected at these sites, respectively. No males were identified in either sample, and poor chromosome morphology did not allow the IIL-1_{ar} inversion to be investigated further (which is why sections 65 and 66 could not be identified) although the polymorphism was seen heterozygously in each of the 7 individuals examined.

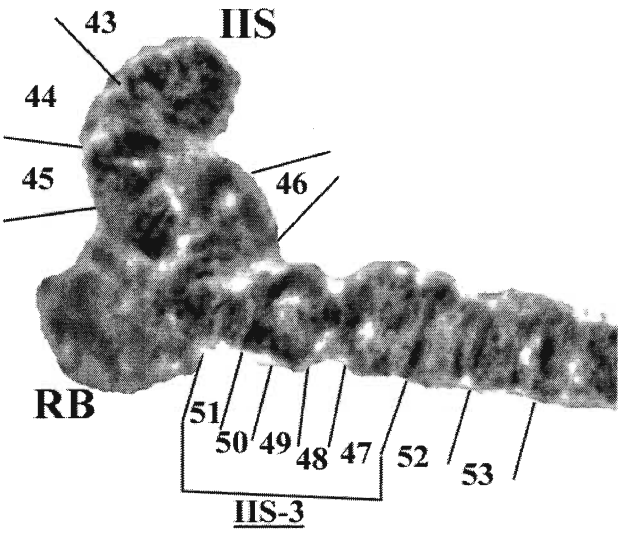

Figure 14: The IIS-3 inversion in *Simulium arlecchinum* (indicated by brackets). RB= Ring of Balbiani.

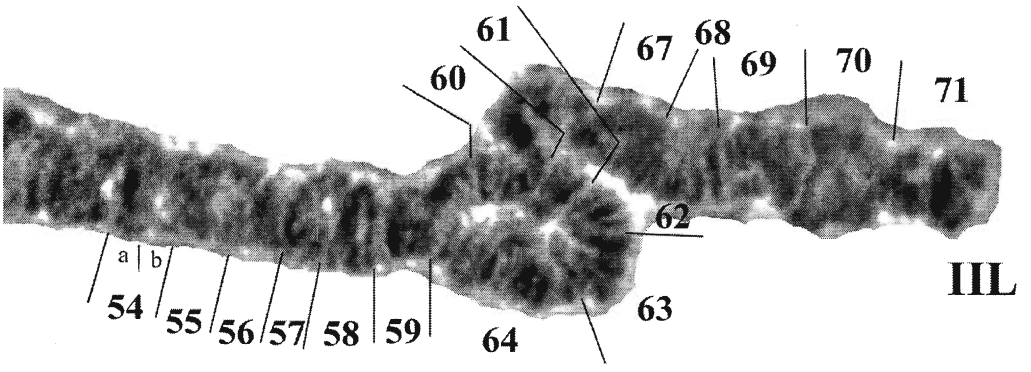

Figure 15: The IIL-1_{ar} inversion polymorphism in *Simulium arlecchinum*.

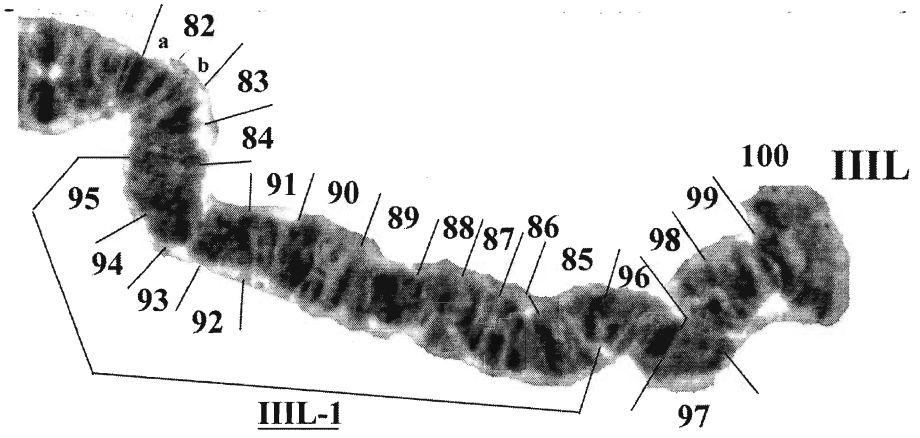

Figure 16: The IIIL-1 inversion in *Simulium arlecchinum* (indicated by brackets).

Simulium exasperans

Chromosome arms IS, IIS, and IIIS were homosequential to the *S. cataractarum* standard. All together, three sites were sampled: 1st Afareaitu Cascade, Jarden Public Vaipahi, and Belvedre Cascade. No fixed inversions were observed, but three floating polymorphisms were discovered (one each in IL, IIL, and IIIL) and warranted further investigation. The three floating inversions were present in individuals in all three sites, and the results of scored, sexed individuals as homozygotes or heterozygotes are given in Tables 7, 8, and 9.

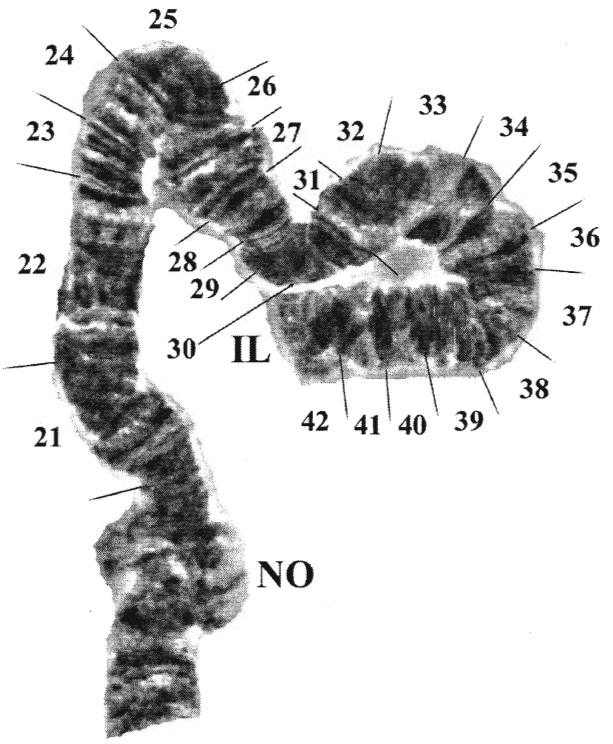

Figure 17: The IL arm of *Simulium exasperans* showing the standard sequence found also in *S. cataractarum*. NO= nucleolar organizer.

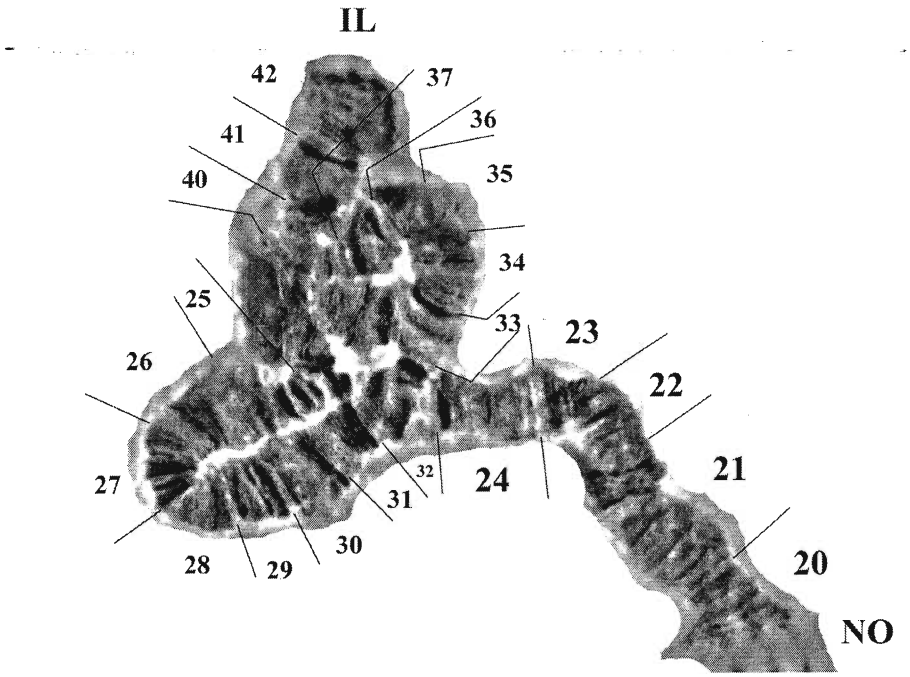

Figure 18: The IL-1_{ex}.2_{ex} complex polymorphism in *Simulium exasperans*. This is representative of a double heterozygote (i.e., IL-1_{ex} s/i .2_{ex} s/i). Inversion breakpoints are shown in Figure 19. NO= nucleolar organizer.

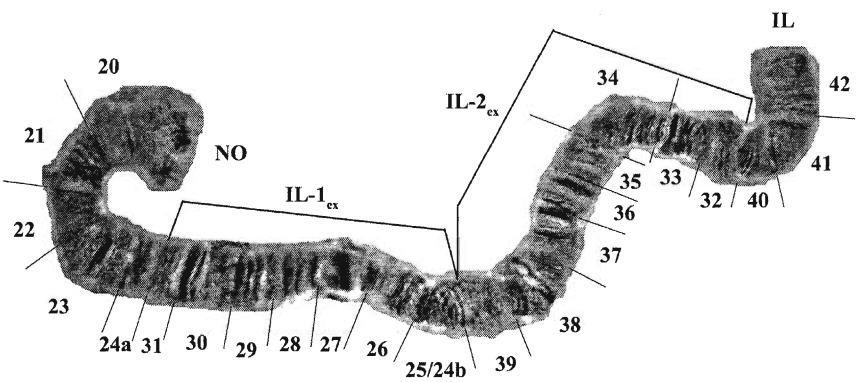

Figure 19: The IL-1_{ex}.2_{ex} complex polymorphism in *Simulum exasperans*. This is representative of a double inverted homozygote (i.e., IL-1_{ex} i/i .2_{ex} i/i). Inversion breakpoints are denoted by brackets. NO= nucleolar organizer.

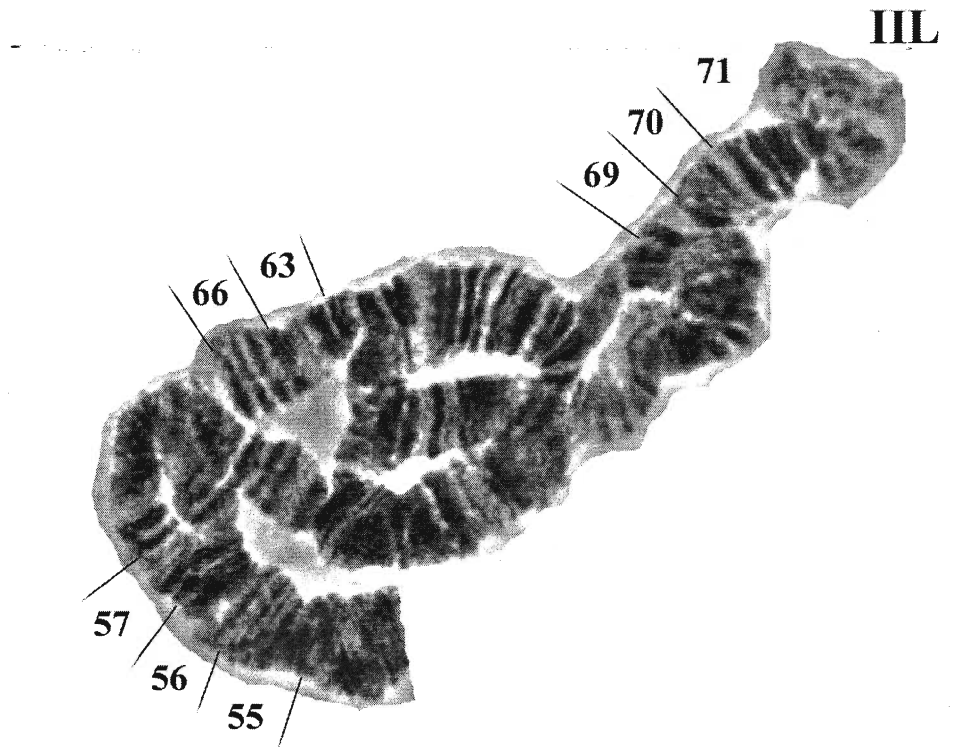

Figure 20: The IIL-1_{ex},2_{ex} complex polymorphism in *Simulium exasperans*. This is representative of a double heterozygote (i.e., IIL-1_{ex} s/i,2_{ex} s/i). Inversion breakpoints are shown in Figure 21.

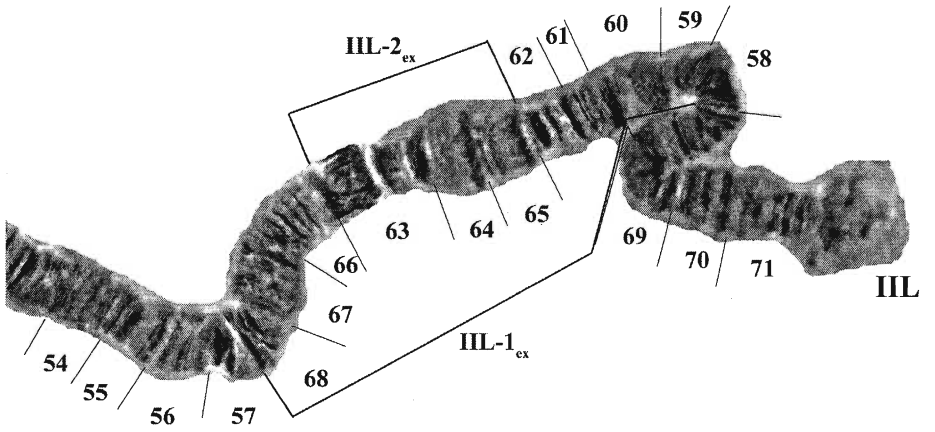

Figure 21: The IIL-1_{ex},2_{ex} complex polymorphism in *Simulium exasperans*. This is representative of a double homozygote (i.e., IIL-1_{ex} i/i,2_{ex} i/i). Inversion breakpoints are denoted by brackets.

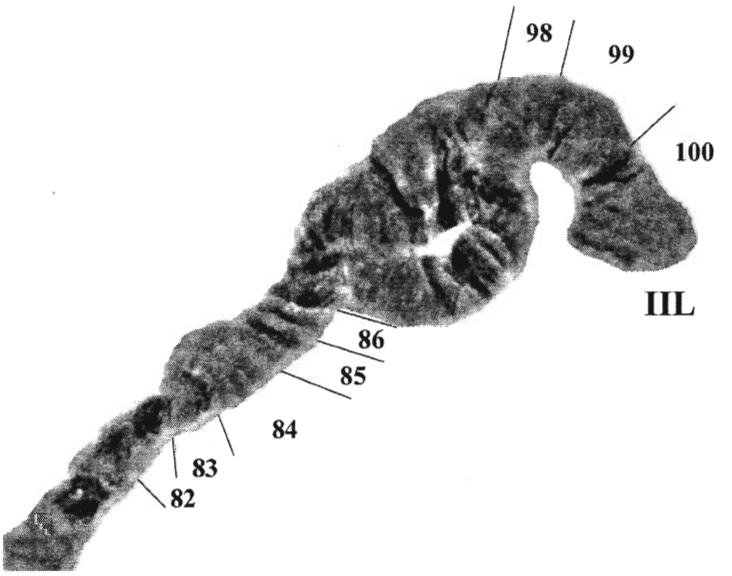

Figure 22: The IIIL-1_{ex} polymorphism in *Simulium exasperans*. This is representative of a heterozygous individual (s/i) for the polymorphism. Inversion breakpoints are shown in Figure 23.

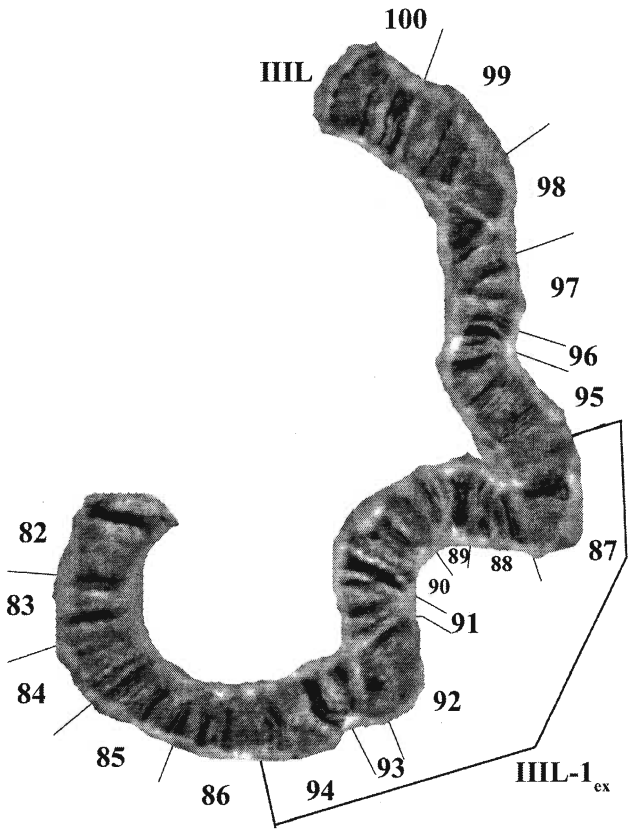

Figure 23: The IIIL-1_{ex} polymorphism in *Simulium exasperans*. This is representative of a homozygous individual (i/i) for the polymorphism.

Table 7: Inversion data from individuals sampled from 1st Afareaitu Cascade 11-11-00. Inversions were scored in comparison to the *S. cataractarum* standard (homozygous standard (s/s), heterozygous standard (s/i), or homozygous inverted (i/i)). The two inversions IL-1_{ex}.2_{ex} and IIL-1_{ex},2_{ex} were always found together, never singularly (i.e., never IL-1_{ex} s/s IL-1_{ex} s/i or i/i. Note: row totals do not equal totals given in Table 5, since not all sexed individuals could be scored for the inversion.

Inversion/Sex	s/s	s/i	i/i
IL-1_{ex}.2_{ex}			
Males	2	1	2
Females	1	1	2
IIL-1_{ex},2_{ex}			
Males	0	4	1
Females	0	4	0
IIIL-1_{ex}			
Males	0	2	3
Females	0	2	2

Table 8: Inversion data from individuals sampled from Jarden Public Vaipahi 9-10-00. Inversions were scored in comparison to the *S. cataractarum* standard (homozygous standard (s/s), heterozygous standard (s/i), or homozygous inverted (i/i)). The two inversions IL-1_{ex}.2_{ex} and IIL-1_{ex},2_{ex} were always found together, never singularly (i.e., never IL-1_{ex} s/s IL-1_{ex} s/i or i/i.

Inversion/Sex	s/s	s/i	i/i
IL-1_{ex}.2_{ex}			
Males	0	0	0
Females	2	1	1
IIL-1_{ex},2_{ex}			
Males	0	0	0
Females	0	4	0
IIIL-1_{ex}			
Males	0	0	0
Females	4	0	0

Table 9: Inversion data from individuals sampled from Belvedre Cascade 11-11-00. Inversions were scored in comparison to the *S. cataractarum* standard (homozygous standard (s/s), heterozygous standard (s/i), or homozygous inverted (i/i)). The two inversions IL-1_{ex}.2_{ex} and IIL-1_{ex},2_{ex} were always found together, never singularly (i.e., never IL-1_{ex} s/s IL-1_{ex} s/i or i/i.

Inversion/Sex	s/s	s/i	i/i
IL-1_{ex}.2_{ex}			
Males	1	1	0
Females	2	1	2
IIL-1_{ex},2_{ex}			
Males	0	0	2
Females	0	2	2
IIIL-1_{ex}			
Males	0	2	0
Females	3	0	1

1st Afareaitu Cascade

Males and females were observed with polymorphisms in all three arms. There was no difference between males and females for the presence of the IL-$1_{ex}.2_{ex}$ rearrangement. The IIL-$1_{ex},2_{ex}$ inversion was equally present in males and females but was only observed heterozygously, thus no sex-linkage was observed for the polymorphism. The IIIL-1_{ex} was also observed somewhat evenly for males and females, but no standard/standard (s/s) individuals were observed (Table 7).

Observed values of homozygotes and heterozygotes of all individuals in the population are given in Table 10, as well as their expected frequencies if the population were randomly mating (using a Hardy-Weinberg calculation). After comparing these values using a G-statistic (Table 10), it was determined that only the IIL-$1_{ex},2_{ex}$ inversion was not in Hardy-Weinberg equilibrium ($G=23.9$, $\alpha=0.05$).

Table 10: Observed values of homozygotes and heterozygotes from individuals from 1st Afareaitu Cascade 11-11-00 for each inversion in comparison to the standard, *S. cataractarum*. Expected frequencies calculated using a Hardy-Weinberg calculation are also presented. Results from a G-statistic (α=0.05, crit.=5.991) are given in parentheses beside each inversion.

Inversion/G-value	s/s	s/i	i/i
IL-1_{ex}.2_{ex} (G=3.2)			
Observed	4	3	5
Expected	2.5	5.9	3.5
IIL-1_{ex},2_{ex} (G=23.9)			
Observed	0	10	1
Expected	2.2	2.7	3.3
IIIL-1_{ex} (G=2.8)			
Observed	0	6	5
Expected	0.8	4.3	5.8

Jarden Public Vaipahi

Few individuals (four females) with good chromosome morphology were available to this particular site. The IL-$1_{ex}.2_{ex}$ inversion was observed both homozygously and heterozygously. The IIL-$1_{ex},2_{ex}$ inversion was only observed heterozygously, and the IIIL-1_{ex} inversion was only observed as homozygous standard. Despite the lack of heterozygotes in sexed individuals, the IIIL-1_{ex} inversion was not fixed, since one unsexed individual was observed with the IIIL-1_{ex} inversion heterozygously. As in the previous site, the observed values of homozygotes and heterozygotes are given in Table 11. After comparing these values using a G-statistic, it was determined that all the inversions except the IIL-$1_{ex},2_{ex}$ polymorphism ($G=8.3$, $\alpha=0.05$) were in Hardy-Weinberg equilibrium.

Table 11: Observed values of homozygotes and heterozygotes from individuals from Jarden Public Vaipahi 9-10-00 for each inversion in comparison to the standard, *S. cataractarum* . Expected frequencies calculated using a Hardy-Weinberg calculation are also presented. Results from a G-statistic (α=0.05, crit.=5.991) are given in parenthesis beside each inversion.

Inversion/G-value	s/s	s/i	i/i
IL-1_{ex}.2_{ex} (G=0.99)			
Observed	3	1	1
Expected	2.5	2.1	0.45
IIL-1_{ex},2_{ex} (G=8.3)			
Observed	0	6	0
Expected	1.5	3.0	1.5
IIIL-1_{ex} (G=0.23)			
Observed	4	1	0
Expected	4.0	0.9	0.05

Belvedre Cascade

Males and females were observed with all three inversions. None of the three inversions were sex-linked. The only other observations were that the IIL-1_{ex},2_{ex} rearrangement was not observed homozygously in males or females, and the IIIL-1_{ex} inversion was not observed heterozygously in females. However, from comparing the observed and expected values of each inversion (Table 12) and using a G-statistic test, it was determined that the population was in Hardy-Weinberg equilibrium for all three inversions.

Table 12: Observed values of homozygotes and heterozygotes from individuals from Belvedre Cascade 11-11-00 for each inversion in comparison to the standard, *S. cataractarum* . Expected frequencies calculated using a Hardy-Weinberg calculation are also presented. Results from a G-statistic (α=0.05, crit.=5.991) are given in parenthesis beside each inversion.

Inversion/Sex	s/s	s/i	i/i
IL-1_{ex}.2_{ex} (G=-4.96)			
Observed	3	2	2
Expected	2.28	4.56	1.35
IIL-1_{ex},2_{ex} (G=4.3)			
Observed	0	3	4
Expected	0.31	0.6	4.4
IIIL-1_{ex} (G=-1.61)			
Observed	3	2	1
Expected	2.67	2.67	0.665

S. dussertorum

Chromosome arms IS, IIS, and IIIS were homosequential to the *S. cataractarum* standard (Table 6a). A fixed inversion was found in IL (IL-2,5,6,7), and two floating inversion polymorphisms were found: IIL-1_{du} and IIIL-1_{du}. Only five individuals were collected from one site, three of which were unidentifiable as male or female. Thus the significance of the two floating inversions was undeterminable.

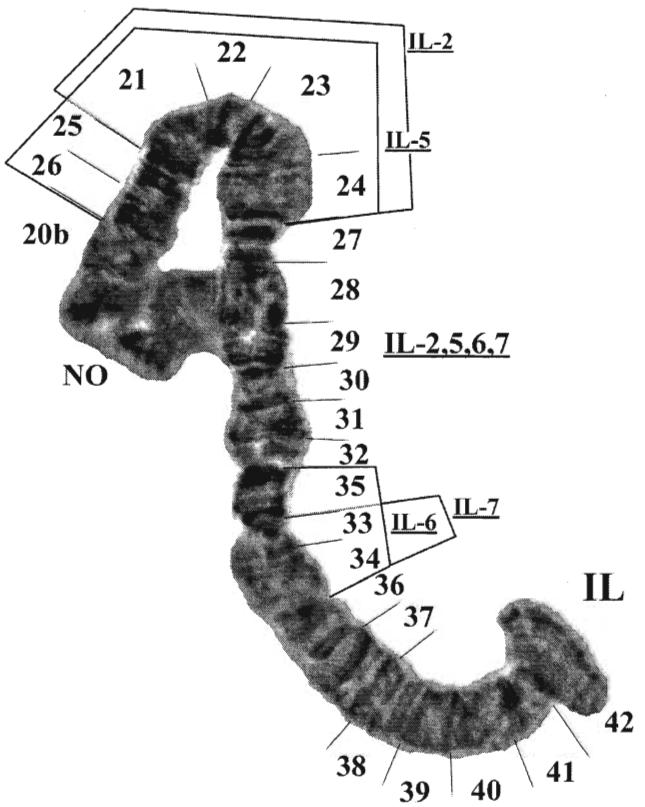

Figure 24: The IL-2,5,6,7 inversion in *Simulium dussertorum* (denoted by brackets). Faded bracket arms indicate where inversions overlap. NO= nucleolar organizer.

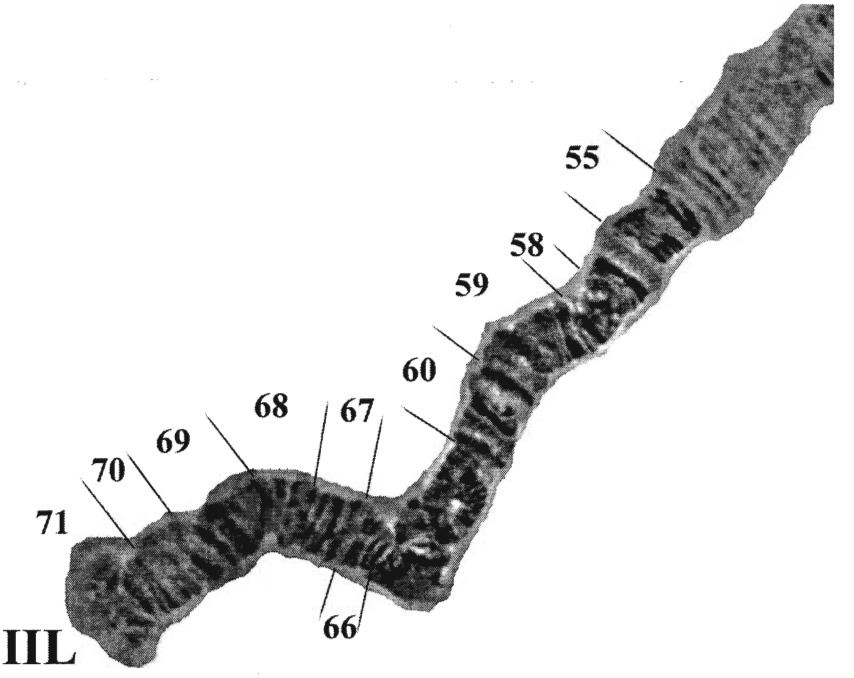

Figure 25: The IIL-1_{du} polymorphism in *Simulium dussertorum*. This is representative of a heterozygous individual (s/i) for the polymorphism. Sections 61-65 and 56-57 could not unambiguously be assigned to the arm.

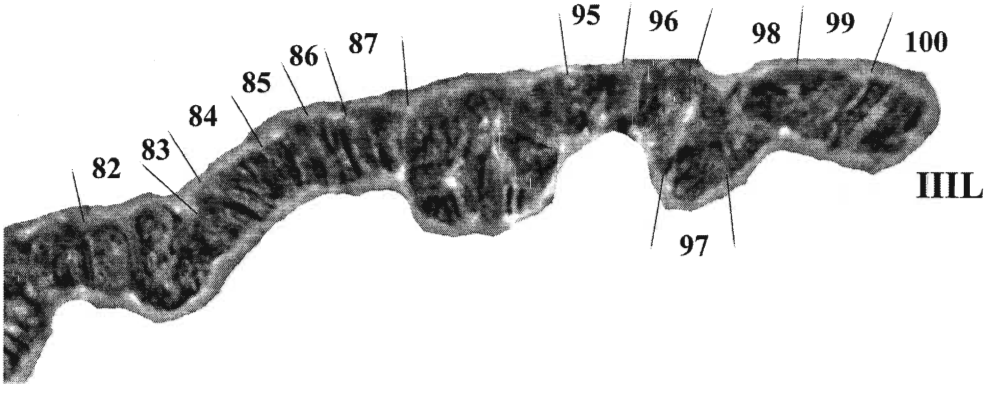

Figure 26: The IIIL-1_{du} polymorphism in *Simulium dussertorum*. This is representative of a heterozygous individual (s/i) for the polymorphism. Sections 88-94 could not unambiguously be assigned to the arm.

S. lotii

Chromosome arm IS and chromosome III were homosequential to the *S. cataractarum* standard (Table 6b). Three fixed inversions were found: IL (IL-1), IIS (IIS-1,2), and IIL (IIL-1). No floating inversion polymorphisms were observed. One site at Haruauta Valley (Raiatea) provided nine usable slides. There were no undifferentiated sex chromosomes.

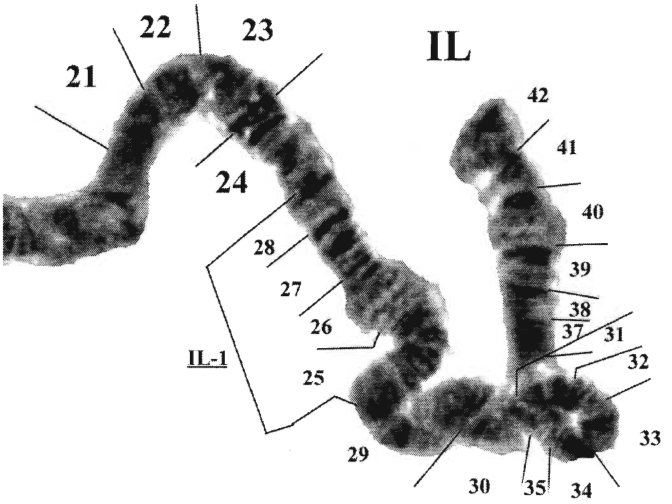

Figure 27: The IL-1 inversion in *Simulium lotii* (denoted by brackets). Looping in this photograph is an artifact of squashing. Section 36 is located under section 31 on the loop.

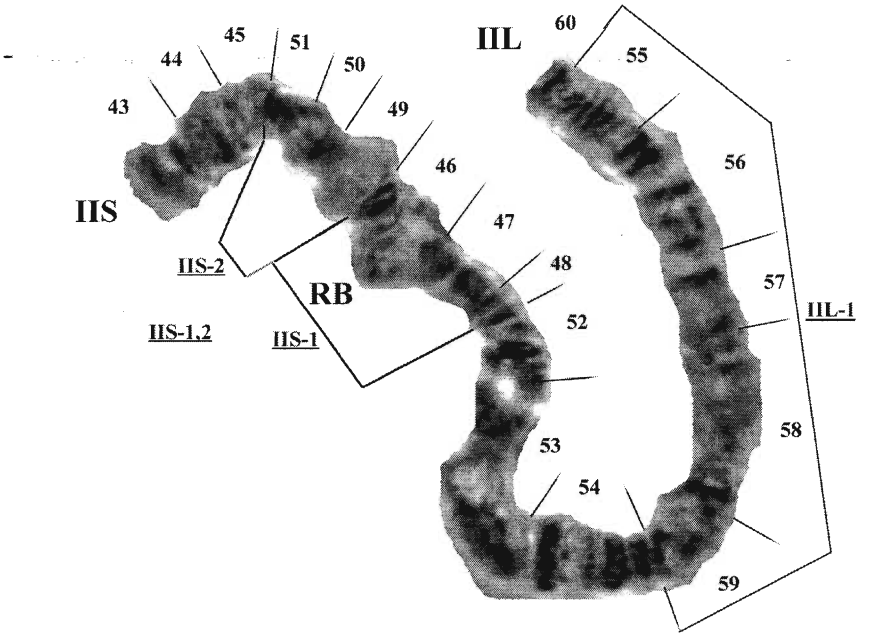

Figure 28: The IIS-1,2 and IIL-1 inversions in *Simulium lotii*. Faded bracket arms indicate where inversions overlap. RB= Ring of Balbiani.

S. hukaense

Chromosome arms IS, IIS, and IIIL were homosequential with the *S. cataractarum* standard (Table 6b). One site at the Hukamae River provided ten usable samples. Three fixed inversions were found: IL (IL-2,3,4), IIL (IIL-2,3,4), and IIIS (IIIS-1). The breakpoints in the IL-2,3,4 inversion were partially the way through a banding region, thus were labeled with letter (a and b) to indicate the proximal and distal ends of the banding region (Figure 29). No floating inversions (autosomal or sex-linked) were observed.

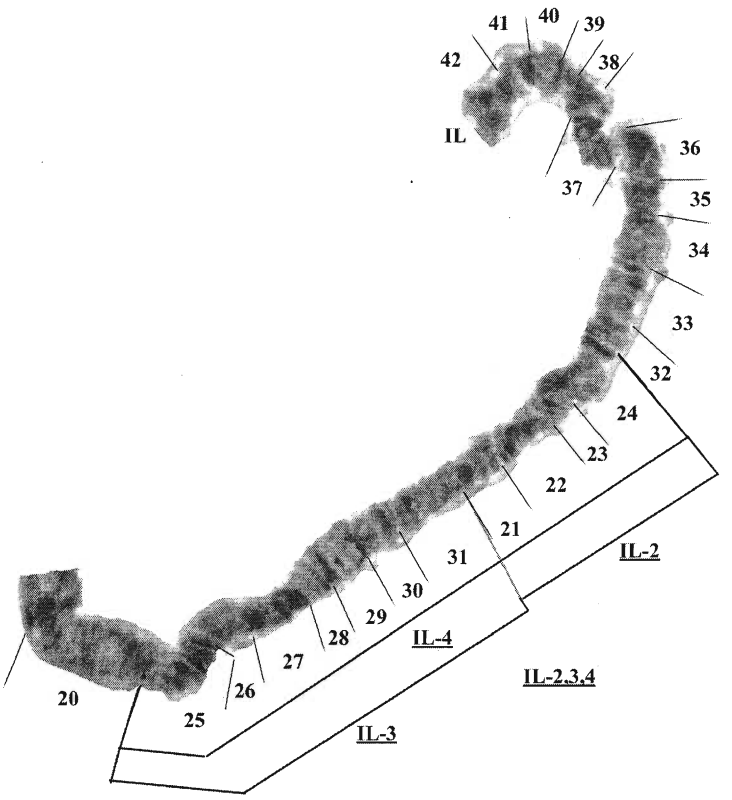

Figure 29: The IL-2,3,4 inversion in *Simulium hukaense* (denoted by brackets). Faded bracket arms indicate where inversions overlap.

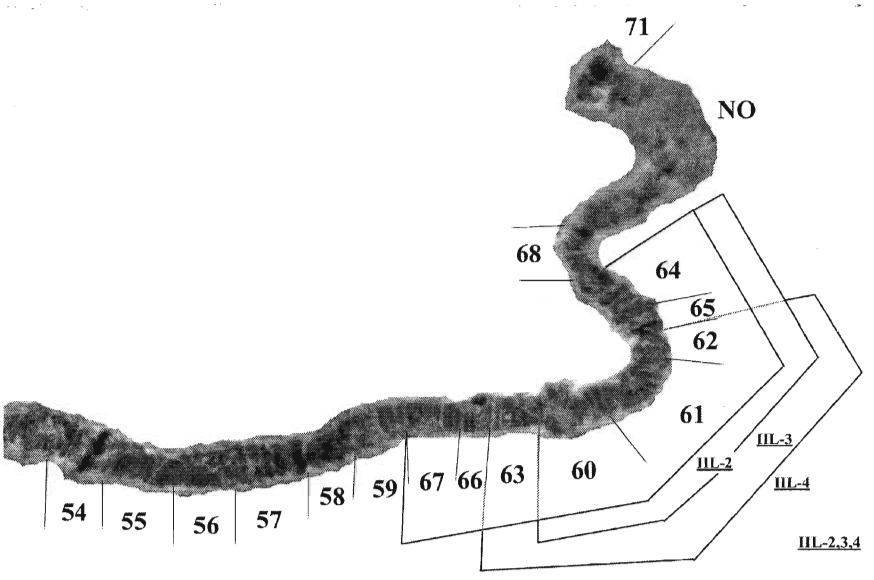

Figure 30: The IIL-2,3,4 inversion in *Simulium hukaense* (denoted by brackets). Faded bracket arms indicate where inversions overlap. NO= nucleolar organizer.

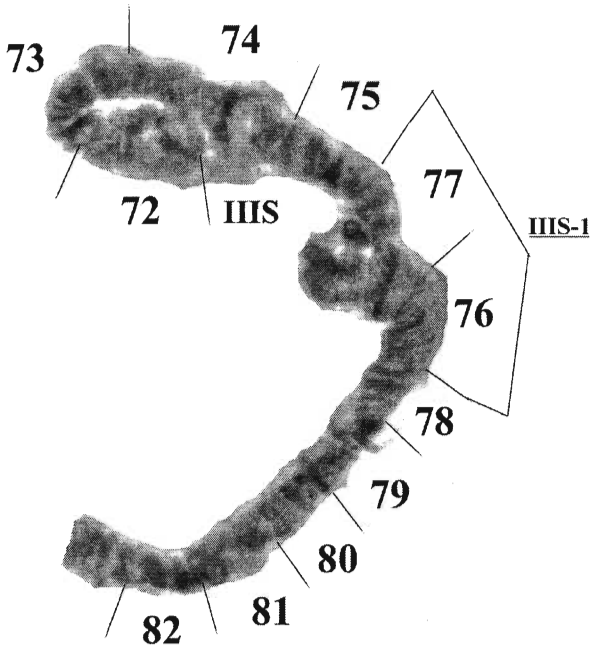

Figure 31: The IIIS-1 inversion in *Simulium hukaense* (denoted by brackets).

S. buissoni

Chromosome arm I and chromosome arms IIL and IIIS were homosequential to the *S. cataractarum* standard. Two fixed inversions were found in IIS (IIS-1), and IIIL (IIIL-2). No floating inversion polymorphisms were observed. One site at Nuku Hiva provided five usable samples. As in the previous species, there were no indications of sex-linked inversions.

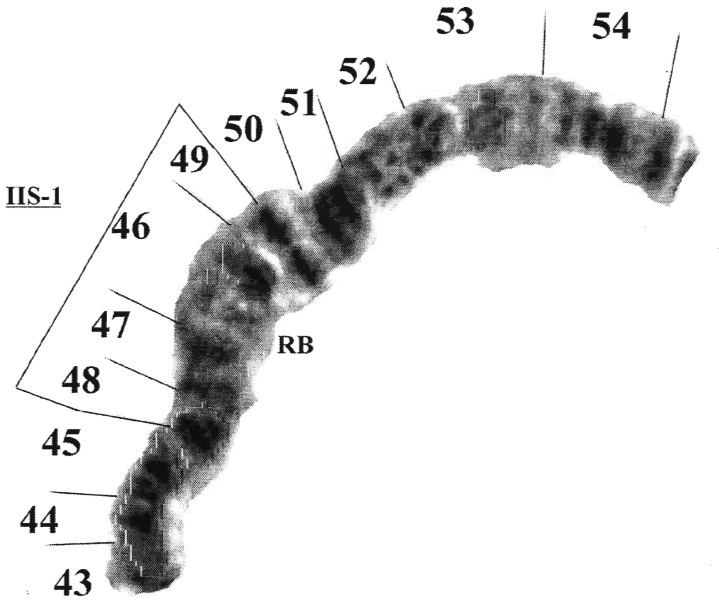

Figure 32: The IIS-1 inversion in *Simulium buissoni* (denoted by brackets). RB= Ring of Balbiani.

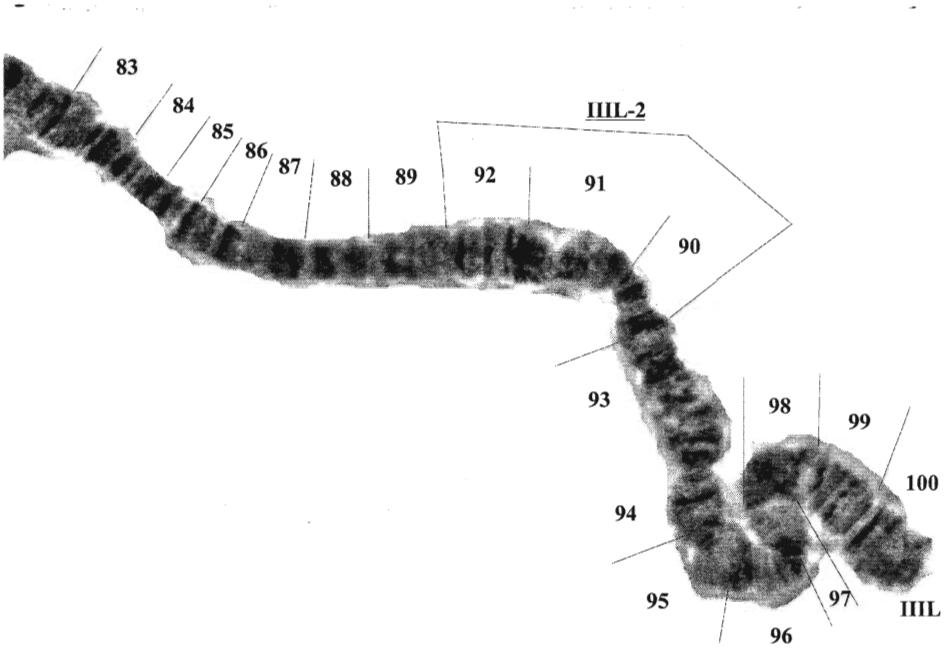

Figure 33: The IIIL-2 inversion in *Simulium buissoni* (denoted by brackets).

S. rurutuense

Chromosome arms I and II were homosequential to the *S. cataractarum* standard. Two fixed inversions were found in chromosome III (IIIS-1 and IIIL-3). Two sites at Above Hauti Tank Stream and Above Vaipapa Stream provided fifteen and nine usable slides respectively. As in the previous species, there were no indications of sex-linked inversions.

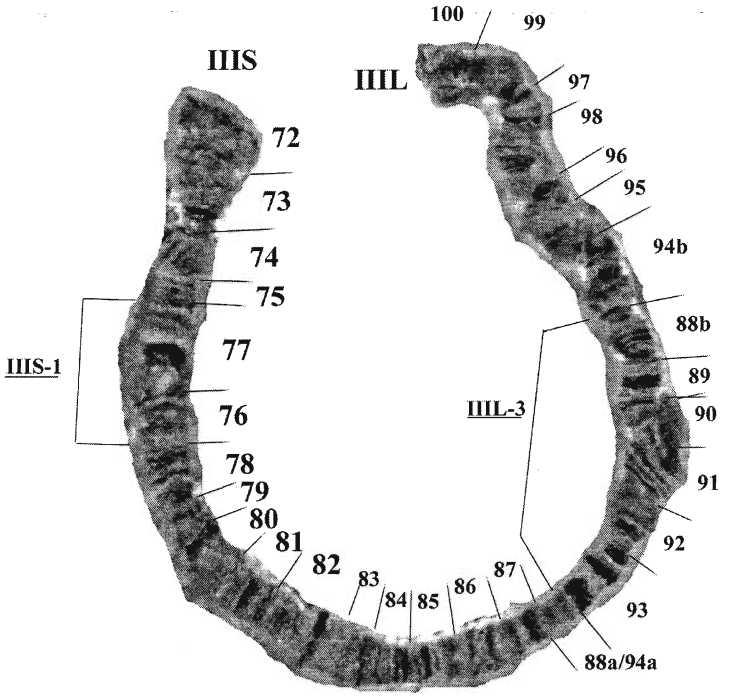

Figure 34: The IIIS-1 and IIIL-3 inversions in *Simulium rurutuense* (denoted by brackets).

Inter-specific Analysis

Chromosome I, Short Arm (IS)

This arm had the most highly conserved banding sequence. The standard IS *S. cataractarum* sequence was shared by the other seven species in this study; there were no polymorphisms in relation to the standard.

The characteristic "stubby" end of this arm was shared by all eight species, as well as the four heavy bands in section 4. The only variation in this arm was in the presence or absence of the nucleolar organizer (NO) landmark. *S. arlecchinum, S.*

exasperans, S. dussertorum, S. cataractarum, and *S. lotii* all possessed the NO at the base of IS in section 20a, whereas *S. hukaense, S. buissoni,* and *S. rurutuense* did not.

Chromosome I, Long Arm (IL)

This arm is one of the most highly rearranged arms in the eight species studied. *S. arlecchinum, S. buissoni,* and *S. rurutuense* all shared the standard *S. cataractarum* sequence. A floating inversion polymorphism in this arm was identified in *S. exasperans* (IL-$1_{ex}.2_{ex}$). *S. lotii* contained a unique fixed inversion in relation to the other species (IL-1 inversion).

S. hukaense and *S. dussertorum* contained the most fixed rearrangements of the species studied. *S. hukaense* was a three-step rearrangement from the *S. cataractarum* standard (IL-2,3,4), whereas *S. dussertorum* was a four-step rearrangement from the standard (IL-2,5,6,7). It was determined that the IL-2 inversion was shared between the *S. hukaensse* and *S. dussertorum.*

This arm was identified by the "flare" at the end of the chromosome arm in section 42.

Chromosome II, Short Arm (IIS)

The standard *S. cataractarum* sequence was shared by four other species: *S. exasperans, S. dussertorum, S. hukaense,* and *S. rurutuense*. The first fixed rearrangement identified in this arm was the IIS-1 inversion in *S. buissoni*. *S. lotii* was one rearrangement step from *S. buissoni*, containing the IIS-1,2 rearrangement. These two inversions indicated that *S. buissoni* and *S. lotii* share the IIS-1 inversion. An

additional fixed inversion was identified in *S. arlecchinum* (IIS-3), which occurred independently of the other rearrangements in IIS.

Chromosomal landmarks in IIS differed in appearance slightly. The major landmark, the Ring of Balbiani (RB), occurs as a puff in section 46 of the standard. The degree of puffing in the RB varied from species to species. *S. arlecchinum, S. exasperans,* and *S. dussertorum* had an expanded puff, whereas in *S. lotii, S. hukaense, S. buissoni,* and *S. rurutuense* the puffing of the RB was not as evident as in the previous species. The three heavy bands in section 51, and the bulge (B) in section 48 were both consistent in appearance, and were both involved in inversions.

Chromosome II, Long Arm (IIL)

This arm contained the most rearrangements, including both fixed and floating inversions. Only two species, *S. buissoni* and *S. rurutuense* shared the standard *S. cataractarum* sequence. Three floating inversion polymorphisms were identified: IIL-1_{ar}, IIL-1_{ex},2_{ex}, and IIL-1_{du}. The first fixed inversion identified in this arm was the IIL-1 inversion, found in *S. lotii*, and was unique to this species. The second fixed rearrangement identified was the IIL-2,3,4 sequence in *S. hukaense*, a three-step rearrangement from the standard. None of the fixed inversions in IIL were shared between the two species that had fixed inversions in this arm.

Unlike the standard, the NO landmark was observed IIL, residing in sections 69 and 70, near the end of the arm in *S. hukaense, S. buissoni,* and *S. rurutuense*. This was in contrast to the presence of the NO in IS in the other species studied. The Parabalbiani landmark in section 64 of IIL was consistently identified as an expanded region in the chromosome arm in all species studied.

Chromosome III, Short Arm (IIIS)

The banding sequence in this arm was highly conserved. One fixed inversion was recognized (IIIS-1) and was shared by two species: *S. hukaense* and *S. rurutuense*. This chromosome was readily identified by its being submetacentric; thus, the short arm was much shorter than the long arm. The end of IIIS usually appeared expanded or flared, which aided in identifying it. The only major landmark, the blister in section 77, was also used to identify IIIS and was involved in the IIIS-1 inversion. This affected the appearance of the blister, appearing opposite in polarity in comparison to the *S. cataractarum* standard (Figure 4E).

Chromosome III, Long Arm (IIIL)

Two species shared the standard *S. cataractarum* sequence: *S. lotii* and *S. hukaense*. Two floating inversions were identified, IIIL-1_{ex} and IIIL-1_{du}. Three fixed inversions were identified, IIIL-1 in *S. arlecchinum,* IIIL-2 in *S. buissoni,* and IIIL-3 in *S. rurutuense*. Each of these inversions differed in breakpoints by 2-4 sections on either side of the inversion, thus it was thought originally that the three species contained the same fixed inversion (Tables 6a and 6b). However, upon further investigation it was determined that the inversions were unique to the species in which they were found.

This chromosome did not contain any major landmarks, but did contain a marker region of three heavy bands that occurred in section 91. This aided in identifying the arm.

Phylogenetic Analysis

Shared Chromosomal Characters

After the interspecific analysis of the seven species under study (eight including the standard *S. cataractarum*), sixteen fixed inversions were found. It was determined that only these were shared among the taxa: IL-2, IIS-1, and IIIS-1 inversions. These three inversions were used as characters for the cytological transformation series and phylogenetic analysis. In addition, the position of the nucleolar organizer (NO) was used as a character since it had two different positions among the taxa (IS or IIL).

Cytological Transformation Series

Figure 35 shows a cytological transformation series based on shared fixed inversions. The standard sequence of *S. cataractarum* is centralized and all other taxa branch from it, since it was the basis of chromosomal comparisons. Two groups are formed: species that contain the nucleolar organizer in IS (IS-NO group), and species that contain the nucleolar orgainzer in IIL (IIL-NO group). *Simulium arlecchinum* and *S. exasperans* branch separately from the standard because they shared no fixed inversions with any other taxa. The rest of the branching is based upon a hypothetical intermediate that contains floating inversions corresponding to IL-2 and IIS-1. The IIS-1 inversion would be carried into *S. buissoni* and *S. lotii* to become fixed, while the IL-2 inversion would be lost in these two species. *Simulium buissoni* and *S. lotii* did not branch together from the standard through the sharing of the IIS-1 inversion because of the position of the nucleolar organizer (IS and IIL respectively). *Simulium hukaense* and *S. rurutuense* branch from a common node through the sharing of the IL-2 inversion. *Simulium*

dussertorum branches off from the hypothetical intermediate, and the IIS-1 inversion is lost, while the IL-2 inversion becomes fixed (as in *S. hukaense*). Unique fixed inversions are also presented in this cytological transformation series for possible future comparisons with related species.

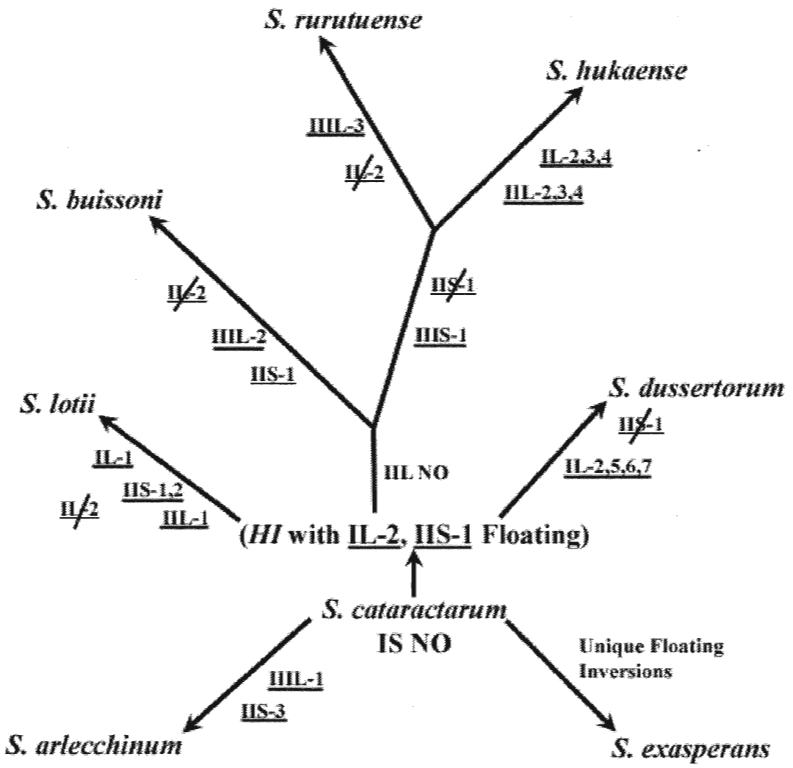

Figure 35: Cytological transformation series (cytophylogeny) of the species of *Inseliellum* under study. Fixed inversions are located on branches and shared fixed inversions are located on branches leading to a split in branching. Lost inversions along lineages are crossed out. *HI* = hypothetical intermediate; NO = nucleolar organizer.

Morphological Phylogeny

For comparative purposes, a morphological phylogeny was created from the data matrix from Craig *et al.* (2001) for the eight species from this study using 28 characters. One most parsimonious tree was found with a tree length of 56, CI of 0.79, RI of 0.75. This tree is shown in Figure 36. Bremer support showed support for the ingroup node, with a value of 6, and the node at *S. cataractarum* and *S. dussertorum*, with a value of 5. The IS-NO group was highly supported in this phylogeny with a decay index of 6.

Simulium neornatipes was resolved as the sister group of the rest of the other taxa. Two main clades were formed, consisting of *S. buissoni* and *S. hukaense* in one, and the rest of the taxa in the second.

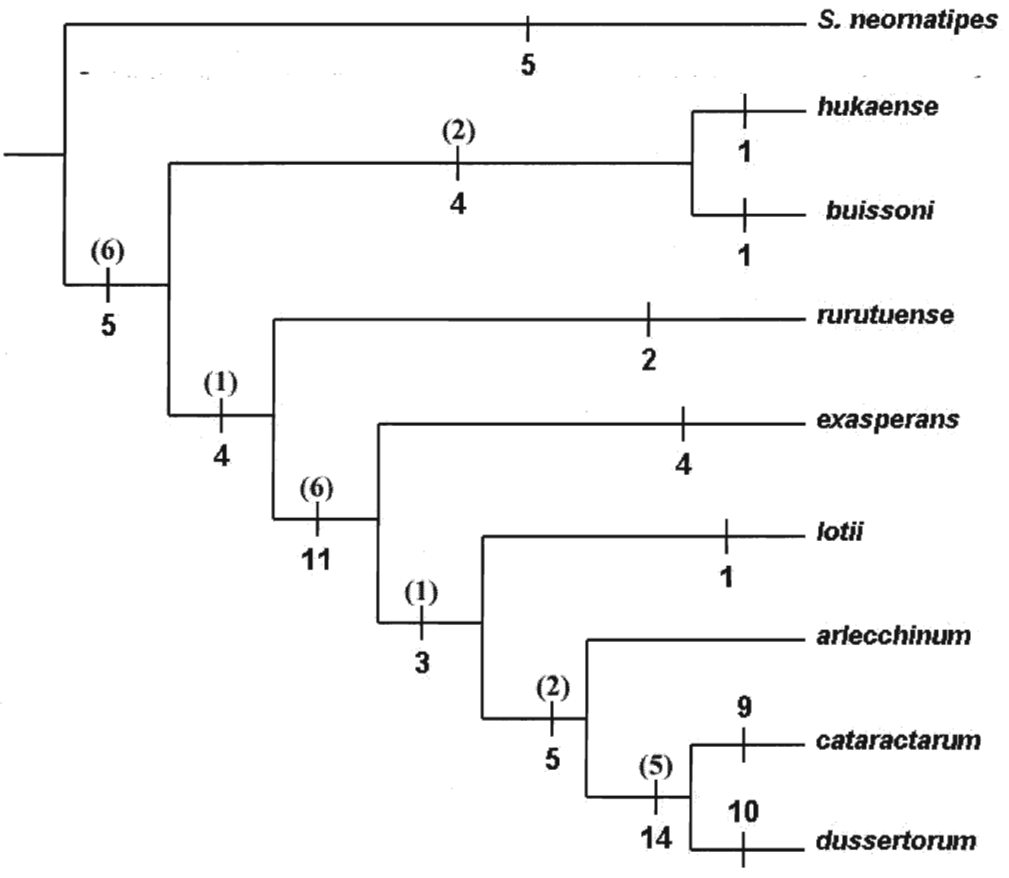

Figure 36: Morphological phylogeny (Tree length 56, CI 0.79, RI 0.75) derived from 1 EPT after heuristic analysis by PAUP of eight species of *Inseliellum*. Vertical lines with values represent the number of character state changes, while numbers in brackets indicate decay indices.

Combined Phylogeny

The combined cytological and morphological data matrix contained 32 characters. By then successively reweighting the NO from 2 to 10, only one most parsimonious tree was found (tree length of 64, CI of 0.78, RI of 0.72, and 67 changes). This tree is shown in Figure 37. Bremer support showed some support for the ingroup node, with a value of 6, and the node at *S. cataractarum* and *S. dussertorum*, with a value of 5. Again the IS-NO group was highly supported, with a decay index of 6.

Simulium neornatipes was resolved as the sister group to the other taxa. This tree was identical to the morphological tree. *Simulium buissoni* and *S. hukaense* were again placed as sister taxa (NO in IIL), but *S. rurutuense* (NO in IIL) was not grouped with these species. *Simulium exasperans, S. lotii, S. arlecchinum, S. cataractarum* and *S. dussertorum* were a monophyletic group, all having the NO in IS.

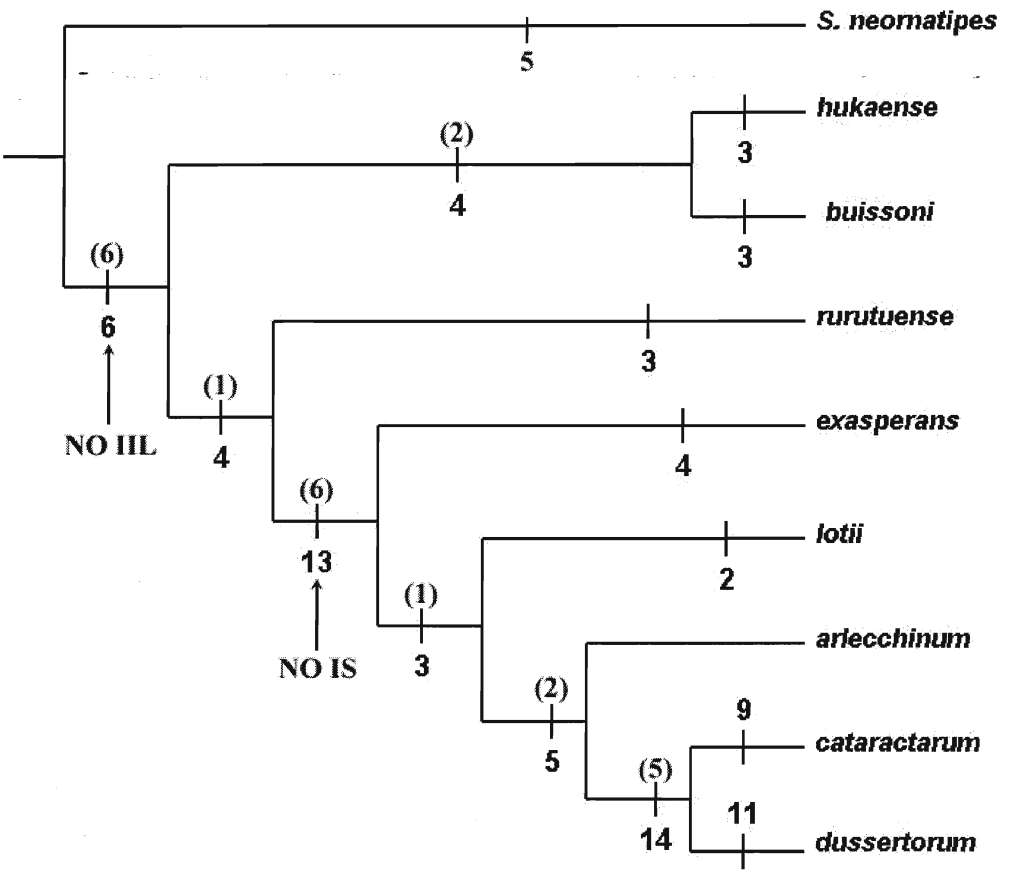

Figure 37: Phylogeny from the combined morphological and chromosomal data (Tree length 64, CI 0.78, RI 0.72) derived from 1 EPT after heuristic analysis by PAUP of eight species of *Inseliellum*. Vertical lines with values represent the number of character state changes, while numbers in brackets indicate decay indices. Arrows indicate nucleolar organizer (NO) position change.

DISCUSSION

Chromosome Maps

The polytene chromosomes of *Simulium arlecchinum, S. exasperans, S. dussertorum, S. lotii, S. hukaense, S. buissoni,* and *S. rurutuense* have been mapped (Figures 14-34). The banding patterns of all species have been compared to the standard maps of *S. cataractarum* (Figures 8-13).

For this study, the chromosomal maps of *S. cataractarum* were chosen as the standard for two reasons: it is in the subgenus *Inseliellum*, and it is the only member of this subgenus that has been previously mapped chromosomally. The term standard does not imply ancestry, but is simply a term used to describe the chromosomal maps to which all taxa under study were compared (i.e., the starting point taken in mapping). Phylogenetically, in relation to the seven species studied here, *S. cataractarum* is not exactly central in the phylogeny on *Inseliellum*. The more central the standard is, the easier it may be to trace chromosomal rearrangements away from the standard, including the standard, and up to the standard.

Sex-Linked Polymorphisms and Other Inversion Polymorphisms

S. exasperans was the only species in this study that provided enough data to investigate the possibility of sex-linked polymorphisms. Three inversion polymorphisms were found: IL-$1_{ex}.2_{ex}$, IIL-$1_{ex},2_{ex}$, and IIIL-1_{ex}. In addition, three sample sites were available for analysis (Tables 7-9). Sex-linkage was not indicated in any of the three sites (i.e., no polymorphic state was limited to one sex only). This indicates that the sex chromosomes of *S. exasperans* are undifferentiated, and there is an X_0Y_0 sex

chromosome system. All inversion polymorphims were in Hardy-Weinberg equilibrium in all sites except for the IIL-1_{ex},2_{ex} inversion at two sites. The IIL-1_{ex},2_{ex} inversion was not in Hardy-Weinberg equilibrium in samples from 1st Afareaitu Cascade (11-11-00) (Table 10) and Jarden Public Vaipahi (9-10-00) (Table 11). This indicates that one or more of the assumptions of Hardy-Weinberg equilibrium are being violated and evolutionary mechanisms are active. Due to the high numbers of heterozygotes in the two populations, it is possible that selection is favouring individuals that contain this polymorphism in the heterozygote state, keeping the two populations out of Hardy-Weinberg equlibrium. Heterozygote advantage has been observed consistently in Hawaiian *Drosophila* and Simuliidae (Rothfels 1979a; Carson 1982b). It can be concluded that individuals from 1st Afareaitu Cascade (11-11-00) and Jarden Public Vaipahi (9-10-00) are considered cytotype A whereas individuals of *S. exasperans* that were sampled from Belvdre Cascade are cytotype B.

Rothfels in Craig (1983) determined that black flies from Tahiti possessed heterogametic females, a trait unique to *Inseliellum*. A cytophylogeny of Tahitian black flies in Rothfels (1989) shows that another species, *S. tahitiense* contains its sex chromosomes in IIL (Z_0W_0 system for heterogamety). The phylogeny of *Inseliellum* (Craig *et al.* 2001) places *S. exasperans* and *S. tahitiense* as sister taxa in the same clade. Perhaps focusing on the IIL arm in these two species may further reveal the sex chromosomes. However, it has rarely been observed that the sex chromosomes have been the same between two or more species (Rothfels 1979b) (Bedo 1984).

In addition to *Simulium exasperans*, *S. arlecchinum* and *S. dussertorum* also contained inversion polymorphisms in IIL (Table 6a). There are no striking similarities

in the breakpoints of the polymorphisms, except that in all three, they tend to range from sections 55 to 59, and sections 65-69. Cytotypes were found in *S. exasperans*, thus further investigation of polymorphims in the IIL arm may also reveal cytotypes in *S. arlecchinum* and *S. dussertorum*.

The polymorphic region of chromsome arm IIL is similar to an example in the Hawaiian *Drosophila*. Carson (1969, 1974, 1982a) presented patterns like the ones seen here based on chromosomal inversion polymorphisms on chromosome 4 of the *Drosophila grimshawi* subgroup. He demonstrated the similarity of polymorphisms among closely related species. The central section of chromosome 4 had polymorphisms in five species. The breakpoints of the polymorphisms only differed from each other by two to four bands. Much of the same chromosome region was involved in each case. Thus this region of the chromosome has selective value when heterozygous and natural selection has favoured and perpetuated randomly-arising inversions in this chromosomal region (Carson 1969). This has also been observed in *Eusimulium aureum*, in which certain regions of the chromosome contained polymorphisms very similar in breakpoints (Leonhardt 1985). This may be the case in *S. exasperans, S. arlecchinum,* and *S. dussertorum*. All three species have polymorphisms in this general region of the chromosome possibly due to the fact that selection has favoured that region in the heterozygous state through stabilizing selection.

An additional polymorphism in *S. arlecchinum* is of particular interest. Rothfels in Craig (1983) reported a cytologically described species named "IIS". Unfortunately, this species was never described morphologically (D. Craig, pers. com.). It was collected in small rivers and streams, from the island of Tahiti-iti (a small offshoot of land

connected to the Southeast corner of Tahiti). It was not reported whether this inversion was floating or fixed. In this study, *S. arlecchinum* was the only species found to have an inversion in the IIS arm (IIS-1_{ar} polymorphism). Since *S. arlecchinum* has been collected from the same habitat types of "IIS" on Tahiti-iti, it is possible that *S. arlecchinum* is the "IIS" that was described by Rothfels in Craig (1983).

Cytological Transformation Series

The cytological transformation series presented in Figure 35 summarizes all of the fixed inversion data, both shared and unique. It illustrates the centrality of the standard, with all taxa independently branching off from it. A hypothetical intermediate (HI) was used to describe the transformation of five taxa. It had to contain IL-2 and IIS-1 but as "floating" inversions. *S. lotii* and *S. dussertorum* share inversions with species that have the nucleolar organizer in IIL, thus the use of the HI prevents the position of the nucleolar organizer evoloving independently twice. The IL-2 and IIIS-1 "floating" inversions in the HI were carried by the *S. rurutuense/hukaense/buissoni* lineage, in which the IL-2 inversion is lost by *S. rurutuense* and *S. buissoni* at the split of *S. hukaense, S. rurutuense,* and *S. buissoni* and was fixed in *S. hukaense* and in *S. dussertorum* (which branches independently from the HI). The IL-2 inversion was also lost by *S. lotii,* which also branched independently from the HI. The IIS-1 inversion was also carried along the *S. rurutuense/hukaense/buissoni* lineage and was lost by *S. rurutuense, S. hukaense,* and *S. dussertorum*. The IIS-1 inversion then became fixed in *S. buissoni* and *S. lotii. Simulium arlecchinum* did not contain any shared fixed inversions, so it branches off of the HI independently. *S. exasperans* also branches independently from the standard, since it only possessed inversion polymorphisms. The taxa with a NO in IIL do share a common

lineage, however, and the taxa with a NO in IS all stem from the standard, which also has a NO in IS. The problem with this cytological transformation series is that there is no directionality, and the majority of the relationships shown are derived from hypothetical intermediates that may or may not be able to be detected in this subgenus. However, this chromosomal phylogeny can now be used for future reference when mapping other species of *Inseliellum*. The NO-IIL group and the NO-IS group that were formed in this transformation series will be discussed further in detail.

The NO-IIL Group

The three species in this group are considered older to the rest of the taxa studied, based on morphological and geological data (Craig *et al.* 2001). In the morphological phylogeny of Craig *et al.* (2001), *S. buissoni* is the sister taxon to *S. hukaense*. In my analysis, *S. hukaense* and *rurutuense* branch off from a common ancestor, due to a shared inversion (IIIS-1). *S. buissoni* may have a closer relation to *S. lotii,* due to a shared inversion in IIS.

The geographical data on these species must be considered, and are summarized in Table 13. *Simulium buissoni* and *S. hukaense* occur on the Marquesas Islands, while *S. rurutuense* is found on Rurutu (Austral Islands) (Craig 1997). All three species have large labral head fans and occupy small streams and rivers. From ecological, and geographical data, two explanations can be made from the positioning of these three species in the cytological transformation series. Since the Marquesas Islands had running water earlier than the Austral islands, a form of *S. hukaense* may have dispersed to Rurutu (approximately 2000 km south-west of the Marquesas Islands). This may have occurred recently, since Rurutu was not above water until 1 Ma. The second possibility

is that *S. hukaense* and *S. rurutuense* share a common ancestor that was between the two island chains (possibly in the Cook Islands or Society Islands) that also contains the IIIS-1 inversion. Chromosomal studies on species (especially from the Cook Islands) are warranted to determine this.

Table 13: Geographical, island, and habitat data on the three species in the NO-IIL group. From Craig (1997; Craig *et al.* 2001).

Species	Island (Age (Myr))	Time of Inhabitance	Habitat Type
S. buissoni	Marquesas- Eiao, Nuku Hiva, Ua Huka (1.33-5.75)	Not earlier than 6 Ma	Streams and small rivers
S. hukaense	Marquesas- Ua Huka, Ua Pao (1.33-5.75)	Not earlier than 6 Ma	Streams and small rivers
S. rurutuense	Austral Islands- Rurutu (0.3-12.8)	1.0 Ma	Streams and small rivers

The NO-IS Group

The five taxa in this group are from the Society Islands (Craig 1997). With the exception of *S. cataractarum* the taxa inhabit small streams and rivers (Table 14) (Craig *et al.* 2001). *Simulium lotii, S. arlecchinum, S. exasperans,* and *S. cataractarum* all have distinct labral head fans, but the fans of *S. dussertorum* are highly reduced, indicating a grazing-type feeding behaviour (Craig *et al.* 2001). Shared inversions with taxa of the NO-IIL group may support the dispersal paths of *Inseliellum*, and will be discussed later.

Table 14: Geographical, island, and habitat data on the five species in the NO-IS group. From Craig (1997; Craig *et al.* 2001).

Species	Island (Age (Myr))	Time of Inhabitance	Habitat Type
S. lotii	Society- Tahaa, Raiatea, Huahine, Moorea, Tahiti (1.95-3.30)	8-10 Ma	Streams and small rivers, cascades, grottos, madicolous
S. arlecchinum	Tahiti (0.25-1.75)	8-10 Ma	Streams and small rivers, cascades, grottos, madicolous
S. exasperans	Moorea, Tahiti (1.20-2.25, 0.25-1.75)	8-10 Ma	Streams and small rivers
S. cataractarum	Moorea, Tahiti (1.20-2.25, 0.25-1.75)	8-10 Ma	Cascades, grottos, madicolous
S. dussertorum	Moorea, Tahiti (1.20-2.25, 0.25-1.75)	8-10 Ma	Streams and small rivers

Morphological Phylogeny

The topology of the phylogeny (using morphological characters) (Figure 36) differs from the full morphological phylogeny of Craig *et al.* (2001) (Appendix E). *Simulium neornatipes* is still the outgroup, but there are some changes in the placement of taxa. *Simulium hukaense* and *S. buissoni* are sister taxa, but *S. rurutuense* is not grouped with them. *S. exasperans* is also placed above *S. lotii, arlecchinum, cataractarum,* and *dussertorum,* whereas in the full morphological phylogeny, *S. lotii* and *S. arlecchinum* are placed above exasperans.

The placement of *Simulium rurutuense* is poorly supported by a decay index of 1, but the placement of *S. exasperans* is highly supported by a decay index of 6. One would expect the topology of this phylogeny to be the same as the full morphological phylogeny of *Inseliellum* Craig *et al.* (2001), since nearly the same data set was used.

The possible reason for the placement of *Simulium rurutuense* may be from the use of eight species in the ingroup analysis. Craig *et al.* (2001) used 45 characters from 40 taxa of *Inseliellum* in the full phylogeny. By only using eight taxa, characters had to be removed from this data matrix because they became uninformative (i.e., no changes in character state). Some of the characters that were not included may have aided in keeping *S. rurutuense* grouped with *S. buissoni* and *S. hukaense* and *S. lotii* and *S. arlecchinum* above *S. exasperans*. Despite the placements observed in the morphological phylogeny, the consistency index (CI) remained high at 0.79.

The placement of *S. buissoni* and *S. hukaense* favours the general trend of sister taxa usually occurring on the same island (Joy and Conn 2001). However, *S. rurutuense* raises questions of dispersal.

Combined Phylogeny

The phylogeny of the combined data sets (Figure 37) is the same as the topology of the morphological phylogeny (Figure 36) and had a CI of 0.78. Despite the reweighting of the nucleolar organizer character, the same most equally parsimonious tree was obtained. The cytological data did not group *Simulium rurutuense* with *S. buissoni* and *S. hukaense,* despite all three taxa having the NO in IIL. However, the position of the NO in IS was a suitable character for the monophyletic grouping of *S. exasperans, S. lotii, S. arlecchinum, S. cataractarum,* and *S. dussertorum* (Figure 37).

Joy and Conn's (2001) combined molecular and morphological data set of various species of *Inseliellum*, resulted in poor support of the ingroup taxon. In the combined phylogeny, the ingroup was highly supported. Thus, the morphological data overwhelmed all other data in the combined phylogeny. The Joy and Conn (2001) grouping of *S. cataractarum* and *S. dussertorum* as a sister taxa, and *S. exasperans* and *S. lotii* as sister taxa agree with the combined phylogeny presented here. However, this is the extent of the comparison, because no other taxa were shared with the study of Joy and Conn (2001).

Shared Fixed Inversions and Complexity

Fixed inversions may be shared, but in nearly all cases of this study, a species did not have a single fixed inversion, but a series of fixed inversions that were arranged in a stepwise fashion from the standard to the form that was observed (i.e., IL-2,3,4 complex inversion in *S. hukaense*). Therefore one of the rearrangement steps may be shared between species, but the overall complexity (number of steps) differed from one complex fixed inversion to the next. Observing shared inversions in *Inseliellum* may lead to possible conclusions about dispersal, since geographical data on the various archipelagos are now available (Craig *et al.* 2001).

Rothfels (1979b) commented on the number of rearrangement steps in fixed inversions. The larger the number of fixed differences (number of rearrangement steps) between two species, the greater presumably the genetic distance or the further back the time of split between two species. The result of this was a frequent relation between cytological distance and taxonomic rank in black fly species.

IL-2 Inversion

This fixed inversion was shared by *S. dussertorum* and *S. hukaense*. Tables 12 and 13 show that these two species are on two different islands, namely *S. dussertorum* on Moorea and Tahiti (Society Islands), and *S. hukaense* on Ua Huka and Ua Pao (Marquesas). Geographically, Moorea and Tahiti are somewhat older islands, and may have supported a black fly fauna longer than the Marquesas (Tables 14 and 15) (Craig *et al.* 2001). Sequence comparisons of these two species showed that in *S. hukaense*, IL was IL-2,3,4, whereas in *S. dussertorum*, IL was IL-2,5,6,7. These two species may have shared a common ancestor that dispersed and over time and formed these two species. However, it cannot be determined by this study if this is the case. This inference is supported by the phylogenetic data (Figures 36 and 37) and is somewhat supported by geographical data, due to the overlapping in ages of the islands the two species inhabit.

IIS-1 Inversion

This inversion is much like the IL-2 inversion. It is shared between *S. buissoni* and *S. lotii*. The island situation of these two species is the same as *S. dussertorum* and *S. hukaense*- *S. buissoni* is found in the Marquesas, and *S. lotii* is found in the Society Islands (Tables 14 and 15). The inversion in *S. buissoni* only had one rearrangement step, IIS-1, whereas in *S. lotii*, there was an additional inversion step in IIS, IIS-1,2. This again may indicate that relative to other members of *Inseliellum*, these two species appear to share a common ancestor. This is supported by phylogenetic data (Figures 36 and 37) and may be supported by geographical data (Figure 38).

IIIS-1 Inversion

This inversion was previously discussed in the cytophylogeny section. *Simulium hukaense* and *S. rurutuense* both shared the IIIS-1 inversion, with no additional rearrangement steps in either species. This sharing of a fixed inversion may indicate a common ancestor between the two species, or possibly *S. hukaense*, due to the extremely young age of the island of Rurutu (<1 Myr.).

Possible Dispersal Routes of the Species of *Inseliellum* Studied

The dispersal route of the *Simulium exasperans* cytotypes can be discussed first. The 1st Afareaitu Cascade is located on Moorea, while the Jarden Public Vaipahi is located on Tahiti. Both islands are very close to each other, only being approximately 21 km apart (Dickinson 1998). It is possible that *S. exasperans* first colonized Moorea, being first described from a collection from the Belvedre Cascade (Craig 1997). The population then expanded into the 1st Afareaitu Cascade, where IIL-1_{ex},2_{ex} polymorphism became prominent in the population (IIL-1_{ex},2_{ex} cytotype). This population could have then provided the founder for the population of *S. exasperans* found at Jarden Public Vaipahi (that also has the IIL-1_{ex},2_{ex} polymorphism in Hardy-Weinberg disequilibrium).

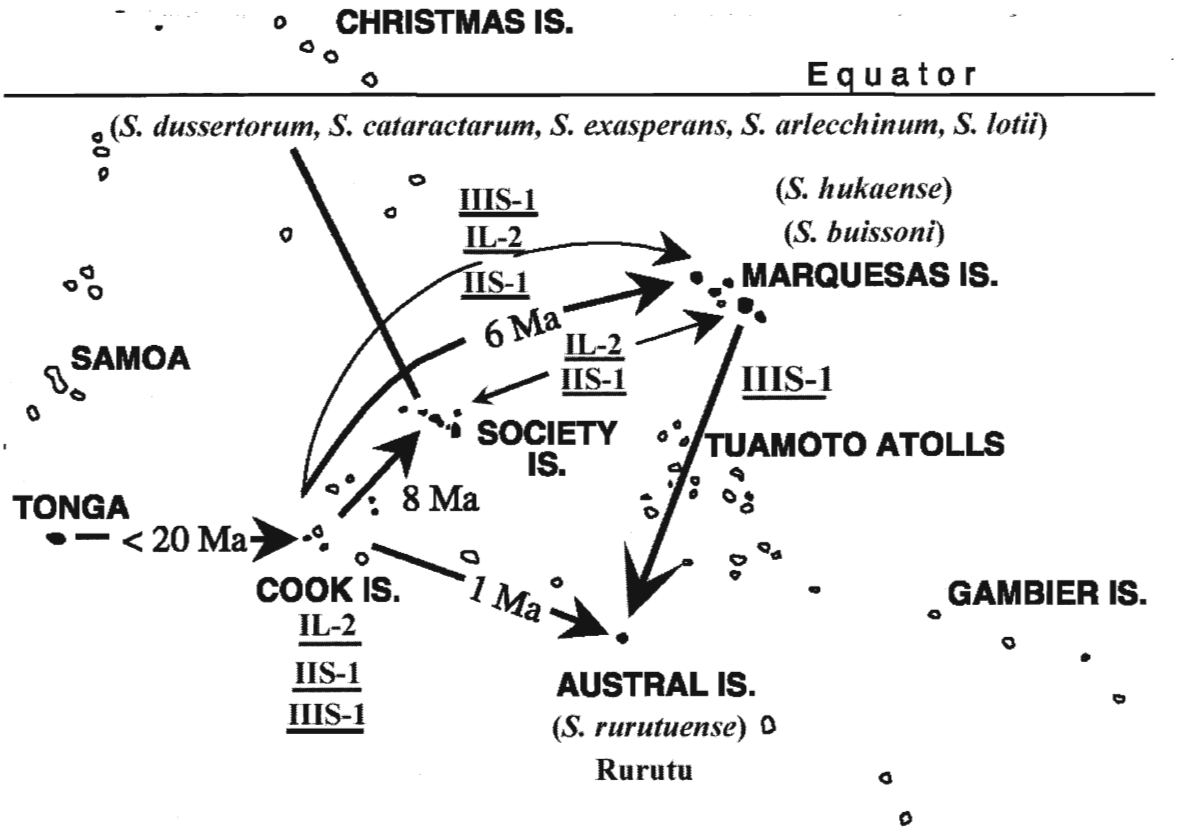

Figure 38: Possible dispersal routes (arrows) of the eight species of *Inseliellum* studied. Shared fixed inversions, as well as oldest possible time of colonization (Ma) are indicated on arrows. A double arrow indicates the possibility of back-dispersal. Arrows west of the Cook Islands are dispersal routes of *Inseliellum* proposed by Craig *et al.* (2001).

Figure 38 shows the possible dispersal routes of the species under study. Theses routes were derived from phylogenetic, cytological, and geological/geographical data. When analyzing possible dispersal routes in archipelagoes, Carson (1982a) suggests that beginning on the youngest island and working backwards towards the oldest is the most effective method, and was effective in determining dispersal routes in Hawaiian *Drosophila*. Thus for *Inseliellum*, this method was adopted.

The youngest island Rurutu (Marquesas) contained *S. rurutuense* (Figure 38). Two possible paths of dispersal are derived for this species. First, colonization by an ancestor from the Cook Islands may have occurred. This would be supported by the existence of a species on the Cook Islands that contained the IIIS-1 inversion and a NO in IIL (Figure 38). The second path is colonization from the Marquesas, with *S. hukaense* being the older species or sharing an extant common ancestor. However, the geographical distance between these two islands is great (approximately 2000 km), and any land masses between the two island chains (Tuamoto Atolls) do not contain any black flies (Craig *et al.* 2001).

The Marquesas Islands are the second youngest in the groups of islands studied, and contain the species *S. hukaense* and *S. buissoni* (Figure 38). Two possible routes of dispersal are possible. The first is again from the Cook Islands. This would require an ancestral species that would contain either both the IL-2 and IIS-1 inversions, or two ancestral species, one containing the IL-2 inversion (ancestral to *S. hukaense* and one containing the IIS-1 inversion (ancestral to *S. buissoni*). If this is the case however, back-dispersal to the Society Islands would have occured, and due to the possible time of colonization for these two island chains, this is poorly supported. The second dispersal route is the same as the previous in the Austral Islands, involving the ancestors of *S. hukaense* and *S. buissoni* to be shared with *S. dussertorum* (shares IL-2 inversion) and *S. lotii* (shares IIS-1 inversion), respectively. This is in congruence with colonization data, and with the proposed dispersal routes of *Inseliellum* by Craig *et al.* (2001) (Figure 1). In both cases of these dispersal routes, a common ancestry in the Cook Islands is required. If species from the Cook Islands have the NO in IS, colonization from the Society Islands

to the Marquesas Islands is favoured. If the NO is found in IIL, colonization from the Marquesas to the Cook Islands is probable. This supported by the phylogenetic data, and Craig *et al.* (2001) in which they postulated that the divergence of the basal lineages and the Polynesian *Inseliellum* took place 20-10 Ma on older Cook Islands.

The last series of islands that contained black flies that were used in this study were the Society Islands, more specifically Moorea and Tahiti. As in the Marquesas, two dispersal routes are possible, but only for *S. dussertorum* and *S. lotii* (due to shared fixed inversion data). The first is similar to the Marquesas- one or two common ancestor(s) that contained the IIS-1 and IL-2 inversions from the Cook Islands that dispersed to the Society Islands. As previously discussed, the second route would stem from the Cook Islands, but an additional step from the younger islands in the Marquesas would have to occur. As for the other Society Island species that were studied, *S. arlecchinum* contained two unique fixed inversions (IIS-3 and IIL-1), and *S. cataractarum* was the standard. *Simulium exasperans* only contained inversion polymorphisms, which was previously discussed. With further chromosomal analysis of more species within the Society Islands and surrounding islands, it may reveal that intra-island speciation has predominated the black fly fauna in the Society Islands, since they contain the highest number of black fly species (Joy and Conn 2001).

Speciation of *Inseliellum*

Two theories of speciation can be discussed in relation to *Inseliellum*. The first is the organizational theory of speciation that has been outlined in Carson (1982b). This speciation theory (via founder effect- extremely small colonizing populations, or founders) was derived from studying Hawaiian *Drosophila* using chromosomal data

(Carson 1984). Thus, applying this theory to *Inseliellum* is justified since *Inseliellum* is found on archipelago, and has been studied chromosomally.

This theory can be condensed into two major steps. The first is the organizational phase, in which an old, phylogenetically balanced gene pool suffers disorganization by stochastic forces. The second is the reorganization phase, in which the gene pool expands, equilibrates, and is maintained in a heterozygous state by stabilizing selection (Carson 1982b).

When considering *Inseliellum*, the disorganization phase can be exemplified by founder events. It has been postulated previously that species of *Inseliellum* on Tahiti originated by a series of founder events (Rothfels 1989). These presence of founder events in *Inseliellum* can be applied to this theory since it entails that speciation can be propagated from a single, sexually reproducing individual from a parent species (founder event) (Carson 1982b). The founder event in this case would involve a mated, blood fed female arriving on a new island via winds or birds. This event would upset selection processes that impinge upon multi-locus systems that control developmental, behavioural, and physiological traits. This would lead to the disorganization of the balanced elements of the ancestral gene pool (Carson 1982b). Accordingly, this situation would be analogous to a reduction in population size, which could occur via environmental factors. According to Craig *et al.* (1995) black flies have "disappeared forever" after a tropical storm in the Marquesas Islands. If events like this have decreased the population sizes of a particular species, low numbers and high inbreeding coefficients would destabalize gene pools, but may also increase genetic variance (Carson 1982b, 1990).

The reorganization phase would involve selection on new, small populations that have low fitness in a new environment. Selection would immediately begin to form new balances. In the case of *Inseliellum*, this would involve the aquatic habitat, whether it be water flow (depth and velocity), substrate type, water pH, and/or water temperature. With selection on the disorganized, variant gene pool, the gene pool will begin to expand in size and will eventually equilibrate through stabilizing selection (Carson 1990).

This theory also fits with *Inseliellum* because the disorganization phase is a very rapid process (less than a few generations). Starting from the Cook Islands, the youngest island that contained a black fly under study was Rurutu, which has been above sea level for 1 million years (Figure 39). Since it only contains one species, that species had to arrive through a single founder that would go under the disorganization and reorganization process. This speciation process would had to have occurred in much less than 1 million years, since the time that Rurutu acquired an aquatic habitat suitable for black flies is much less.

The second theory of speciation thats can be applied to *Inseliellum* entails sympatric speciation. Sympatric speciation involves the development of genetic differences in an initially randomly mating population that share that results in reproductive isolation (Futuyma 1998). Rothfels (1989) applied this theory as a mode of black fly speciation from studies on black fly polytene chromosomes. However, this theory is more applicable to intra-island speciation than to inter-island speciation, since populations on the islands are confined by geography. As far as *Inseliellum* is concerned, once again the species could originate on islands by one or more founder events (Rothfels 1989). Following this event however, over time sibling species could form under

sympatry, and diverge and inhabit other parts of the island, or disperse to another island. To strengthen this, sibling species would have to be identified between two islands (inter-island), ensuring that sibling species under sympatric speciation occurred on one island, and then a sibling dispersed to a new island (Rothfels 1989). Unfortunately, no sibling species were found in this study, and Rothfels (1989) based much of this theory on black flies that contained sibling species with differentiated sex chromosomes. *Simulium tahitiense* and *S. oviceps* were determined to have heterogametic females (Rothfels 1989), but unfortunately it was determined that the species in this study had undifferentiated chromosomes. However, in *S. exasperans*, cytotypes were observed, IIL-1_{ex},2_{ex} A and IIL-1_{ex},2_{ex} B. Two populations containing the IIL-1_{ex} B cytotype were found on Moorea and Tahiti. Cytotypes are almost sibling species, with their inversions being polymorphic, not fixed. In regarding *S. exasperans*, it may be possible that the IIL-1_{ex},2_{ex} A cytotype (Moorea) may have been the founder in the ensuing population of the cytotype A population on Tahiti. This instance does not fit the theory completely, but it is probable despite the fact that the two populations are not sibling species.

Both theories however can be used together and be applied to *Inseliellum*. The organizational theory of speciation explains inter-island speciation well through founder events, and the reorganization of the gene pool that is brought to a stabilized state through selection. The sympatric speciation theory covers both ends of the spectrum- it can take the speciation process a step further focusing on small populations that begin to diverge through the presence of sibling species under sympatry (intra-island speciation), but it also provided means to prove this theory by the existence of a sibling species between two islands (inter-island speciation). Using both of these theories, they provided a basis

for the suggestion of not only relationships between species, but also dispersal and founder events in *Inseliellum.*

Conclusions

From the chromosomal data alone, various conclusions can be made. The first is that in all taxa studied, there were no polymorphisms that indicated sex-linkage, thus all taxa have an undifferentiated sex chromosome system. Another is that the IIL-1.2_{ex} A cytotype in *S. exasperans* acted as a founder for two populations. The first was a population in Moorea that arose from the original population on Moorea, and the second is a population on the adjacent island of Tahiti, which also contained the IIL-1.2_{ex} A cytotype.

From phylogenetic data, it can be hypothesized that dispersal of the taxa under study began on the Cook Islands, and colonization of the Society Islands or Marquesas Islands followed. The colonization of the Society Islands may be preferred, since they are geologically older than the Marquesas, and have been able to support a black fly fauna longer. However, phylogenetic data indicates colonization of the Marquesas Islands first. The Austral Islands has been recently colonized, with the founder of *S. rurutuense* arriving from a NO-IL group ancestor.

The *Inseliellum* system has many parallelisms to the Hawaiian *Drosophila* system by chromosomal inversion data and in the fact that colonization has most likely occurred from jumping from an older island to a new island as they are being formed along the archipelago. These island jumping events contain a founder individual, most likely female and mated. The founding population goes through a disorganization phase and possible bottleneck in the formation of a new species that is adapted to the new habitat.

In *Inseliellum*, this has occurred between islands, but also within islands, with individuals founding new habitats and rapidly speciating. This is exemplified by the large number of species found on the island of Tahiti. Thus inter-island speciation has allowed for the successful colonization of *Inseliellum* to various islands across the South Pacific, but it is intra-island speciation that created the diversity in habitat and form that is observed in this subgenus.

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APPENDICES

Appendix A

Recipe for Carnoy's Fixative (Hunter and Connolly 1986)

Add 3 parts absolute alcohol to 1 part glacial acetic acid (3:1 ratio)

Appendix B

Recipe for Feulgen Stain (Humason 1967)

1. 1 gm of basic fuchsin added to 200 mL of pre-heated distilled water at 80°C
2. Cool to 60°C, add 2 g of potassium metabisulphite
3. Cool to 50°C, add 10 mL 1N HCl
4. Store in a dark fridge over night
5. The next day, add 1 spoonful of Norit (decolourizing carbon), shake and filter

Feulgen stain is now clear, and should be kept in the fridge in the dark

Appendix C

Recipe for Sulfur Dioxide Water (SO_2 water)

1. 200 mL of distilled water
2. 1 gm of potassium bisulphite
3. 10 mL of 1 N HCl
4. mix thoroughly and put in a tightly stoppered bottle

5. store on shelf

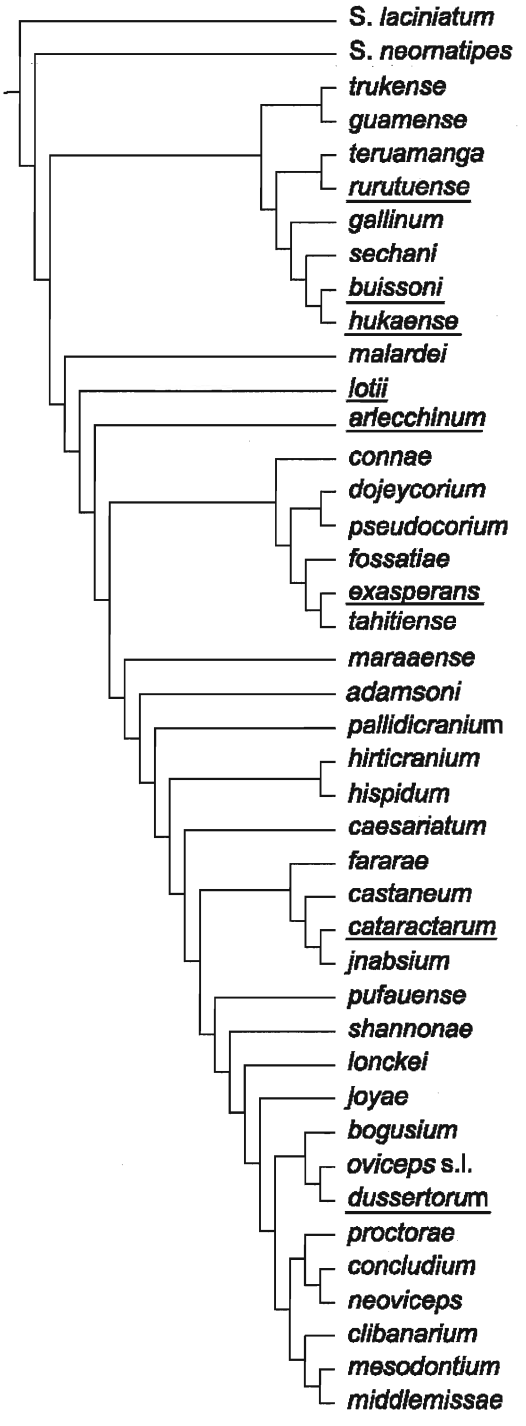
Appendix D

Recipe For 2% aceto-carmine

1. 55 mL distilled water
2. 45 mL acetic acid
3. 5 gms carmine
4. Boil solution for 15 minutes, cool, and filter
5. Add 12 mL of solution to 18 mL for 2% solution

Appendix E

Full Morphological Phylogeny of *Inseliellum* (Craig *et al.* 2001)
Note: Taxa that were used in this study are underlined.


S. laciniatum
S. neornatipes
trukense
guamense
teruamanga
rurutuense
gallinum
sechani
buissoni
hukaense
malardei
lotii
arlecchinum
connae
dojeycorium
pseudocorium
fossatiae
exasperans
tahitiense
maraaense
adamsoni
pallidicranium
hirticranium
hispidum
caesariatum
fararae
castaneum
cataractarum
jnabsium
pufauense
shannonae
lonckei
joyae
bogusium
oviceps s.l.
dussertorum
proctorae
concludium
neoviceps
clibanarium
mesodontium
middlemissae